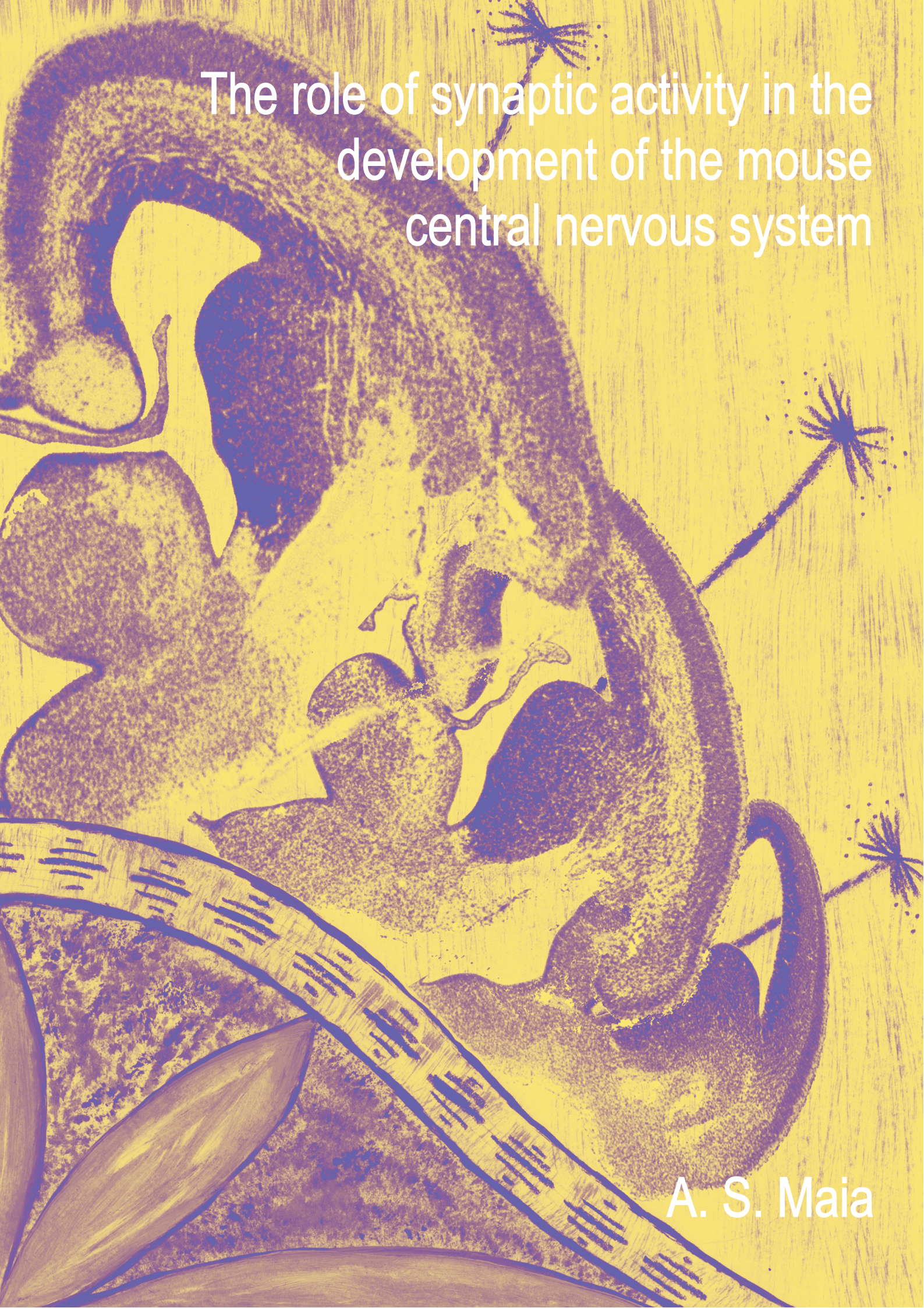
The role of synaptic activity in the development of the mouse central nervous system



A. S. Maia

The role of synaptic activity in the development of the mouse central nervous system

De rol van synaptische activiteit bij de ontwikkeling van het centrale zenuwstelsel van de muis

(met een samenvating in het Nederlands)
(avec un résumé en français)
(con uno resumen en español)
(com um resumo em português)

proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W. H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op woensdag 13 juni 2001 des ochtends te 10.30 uur

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Le véritable lieu de naissance est celui où l'on a porté pour la première fois un coup d'oeil intelligent sur soi-même: mes premières patries ont été des livres.

Marguerite Yourcenar
In "Mémoires d'Hadrien"

À Eurides
Pour Tony
To Declan

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chapter 1

INTRODUCTION

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1) Preface

In this thesis the role that synaptic activity plays in development and maintenance of neuronal connectivity in the brain is investigated. In this introductory chapter, I will review some of the most relevant background literature related to this research project.

The brain is the most complex organ of an animal and consists of approximately 1012 neurones (in humans), which are connected with other neurones in some cases with as many as thousands of functional contacts (synapses). This connectivity enables the brain to control activities such as motor acts as well as more complex processes such as learning and memory. It is now clear that learning is based on molecular and synaptic events, leading to the acquisition and storage of new information in the neuronal networks, probably by a similar process in dissimilar species (see for instance Dudai et al., 1976; Castellucci et al., 1978; Bailey et al., 1996; Lamprecht et al., 1997).

Brain functions are based on networks of communicating cell groups. To mature and function correctly these networks form and eliminate synapses. Synapse formation starts during embryonic development but also occurs in adult animals (Wenzel et al., 1980; Chen and Hillman, 1982). Synapse elimination during synapse refinement (elimination of existing synapses and forming new ones) occurs largely postnatally. Synapse refinement is well characterised for the neuromuscular junction (NMJ) (for review see Lichtman and Colman, 2000) and reported, though less well characterised, during postnatal maturation of the visual system (for review see Katz and Shatz, 1996). To understand how the brain works, it is essential to know how synapses are formed and eliminated. Next I will review some of the critical molecular and morphological events that govern synaptic development, function and maintenance in the nervous system.

2) Brain strategy for development

During central nervous system (CNS) development, genes have a temporal and spatial expression pattern (see for instance Wen et al., 1998), which accounts for neurogenesis and neuronal differentiation. During development, the brain co-ordinates several extremely complex events such as neurogenesis, gliogenesis, cell proliferation, cell migration, axonal outgrowth/path finding, synaptogenesis and cell death. Different communication strategies are employed to co-ordinate these events, for instance the use of secreted molecules, membrane proteins, gap junctions and synapses.

2.1) Secreted molecules

Exocytosis is a method used throughout the whole body for information exchange. Embryonic cells synthesise and release different types of molecules. These molecules can form a chemical gradient in the embryo's body, which in turn may induce expression of specific genes, for instance during cell differentiation. The expression of a set of genes by the target cells depends on the nature and the concentration of the secreted molecule, as well as the availability of the proper receptor on the target cells. Often gradients of several molecules occur simultaneously and intermingle, in order to fine tune gene expression (Barth et al., 1999; Kioussi et al., 1999). Hormones are also released molecules and can be transported over long distances to target cells. They can, for instance, control target cell development. This communication strategy is used for organogenesis and in the brain for cell migration, axon guidance and neurone survival or death.

Many molecules are released from secretory vesicles through the fusion of these vesicles with the plasma membranes. The protein machinery involved in this exocytosis is rather well characterised and is largely conserved from yeast to neurones (Bennett and Scheller, 1993). Likewise, it is becoming clear that neuronal and endocrine cells have similar exocytosis machinery (Bindra et al., 1993; Jacobsson et al., 1994; Majo et al., 1998)

2.2) Membrane proteins

Cell-cell contact is also used to exchange information during embryonic development. This happens through several membrane protein families (for review see Artigiani et al., 1999; Jones and Jones, 2000). The extracellular domain recognises a specific ligand and subsequently the cytoplasmic tail triggers a cascade of second messengers. This strategy is used for axon guidance, cell migration and synapse formation. For details on membrane proteins in synapse formation see 3.1.2 of this chapter.

2.3) Gap junctions

Gap junctions are specialised cell contacts, which permit the diffusion of ions and small molecules up to about 1kDa (Simpson et al., 1977). A gap junction consists of two transmembrane hemichannels or connexons, one from each of the two cells. Each connexon is a complex of six proteins called connexins. These connexins belong to a multigene family. Connexins have primary sequence and membrane topology similarities (Stauffer and Unwin, 1992). Thus, gap junction-linked cells have the capacity to influence each other through electrical activity or signalling molecules (Kandler and Katz, 1998).

These junctions are a common feature during development and are particularly abundant in certain developmental stages. They are involved in neurulation, neuronal differentiation, migration and axon guidance (Fulton, 1995).

2.4) Synapses

The synapse is an asymmetric, specialised cell contact composed of a pre-synaptic element containing synaptic vesicles, an electron dense active zone (containing a large amount of intrasynaptic protein complexes) and a post-synaptic element. Synaptic vesicles fuse at the active zone and release neurotransmitters into the synaptic cleft. Neurotransmitters bind to the pre- and postsynaptic membrane receptors, inducing a cellular response, and have been suggested to be morphogenetic molecules for embryo development (Lauder, 1988; Buznikov et al., 1996). If this is the case, they must be released via a protein machinery different to that used by synaptic vesicle because animals unable to fuse their synaptic vesicles developed normally (Augustin et al., 1999; Verhage et al., 2000; Washbourne et al., 2000). The role of synaptic release in synapse formation and maintenance is controversial (see below). Consequently, the role of synaptic release in brain development is largely unknown.

3) Structural plasticity of synapses

Synapses are crucial for the adult brain's functions and are plastic, i.e. they are constantly being formed, eliminated and/or reshaped. For instance, the numbers of synapses per unit of neuropil in the stratum radiatum (CA1) of the hippocampus significantly increased in rats after they have been subjected to a

training period (Wenzel et al., 1980). There is evidence that long-term potentiation of synaptic efficacy induces synaptic spine changes in the hippocampus (Engert and Bonhoeffer, 1999; Toni et al., 1999). It was also shown that synaptic structures undergo a conformational change after a treatment to induce olfactory memory formation in female mice (Matsuoka et al., 1997).

3.1) Synapse formation

3.1.1) Morphology of synapse formation

Hinds and Hinds (1976) suggested that in the mouse olfactory lobe, a poorly developed electron dense postsynaptic membrane specialisation is the first morphological step towards the future synapse (Fig.1A). In their studies of axodendritic synapses, synaptic vesicles arrive before the post-synaptic membrane thickens. In olfactory lobe dendrodendritic synapses however, the postsynaptic membrane first thickens and later synaptic vesicles are recruited. In both these cases, asymmetric synapses (which have broader electron dense material at the postsynaptic membrane) are formed. Blue and Parnavelas (1983) also suggested that in the rat visual cortex, poorly developed electron dense membranes are the origin of asymmetric and symmetric synapses (which have electron dense material similarly broad in both membranes) (Fig.1B). For symmetric synapses, the pre- and postsynaptic membranes do not further thicken into electron dense membranes and few synaptic vesicles arrive at the presynaptic membrane. However for asymmetric synapses, the postsynaptic membrane first thickens and then the synaptic vesicles are recruited. Thus is it possible that synapse formation steps are specific for each neurone type within each brain area.

Rees et al. (1976) described the events following synaptic contact between superior cervical ganglia and spinal cord neurones from *in vitro* sequence-time lapse studies (Fig.2). Prior to contact, the growth cone has several filopodia. One filopodium establishes physical contact with a target neurone. This contact is a multiple punctuated area. After contact, the Golgi apparatus of the target neurone hypertrophies and increases the production of coated vesicles. The latter will transport the thickening material for the postsynaptic element. After these events, the contacting filopodium changes its form, the other non-contacting filopodia disappear and synaptic vesicles arrive. The synapse matures by increasing synaptic vesicle numbers, appearance of the synaptic cleft, development of presynaptic specialisation and disappearance of pleomorphic vesicles and/or lysosome-like structures. This time sequence study describes steps of synapse formation and maturation but still does not elucidate how CNS synapses develop *in vivo.*

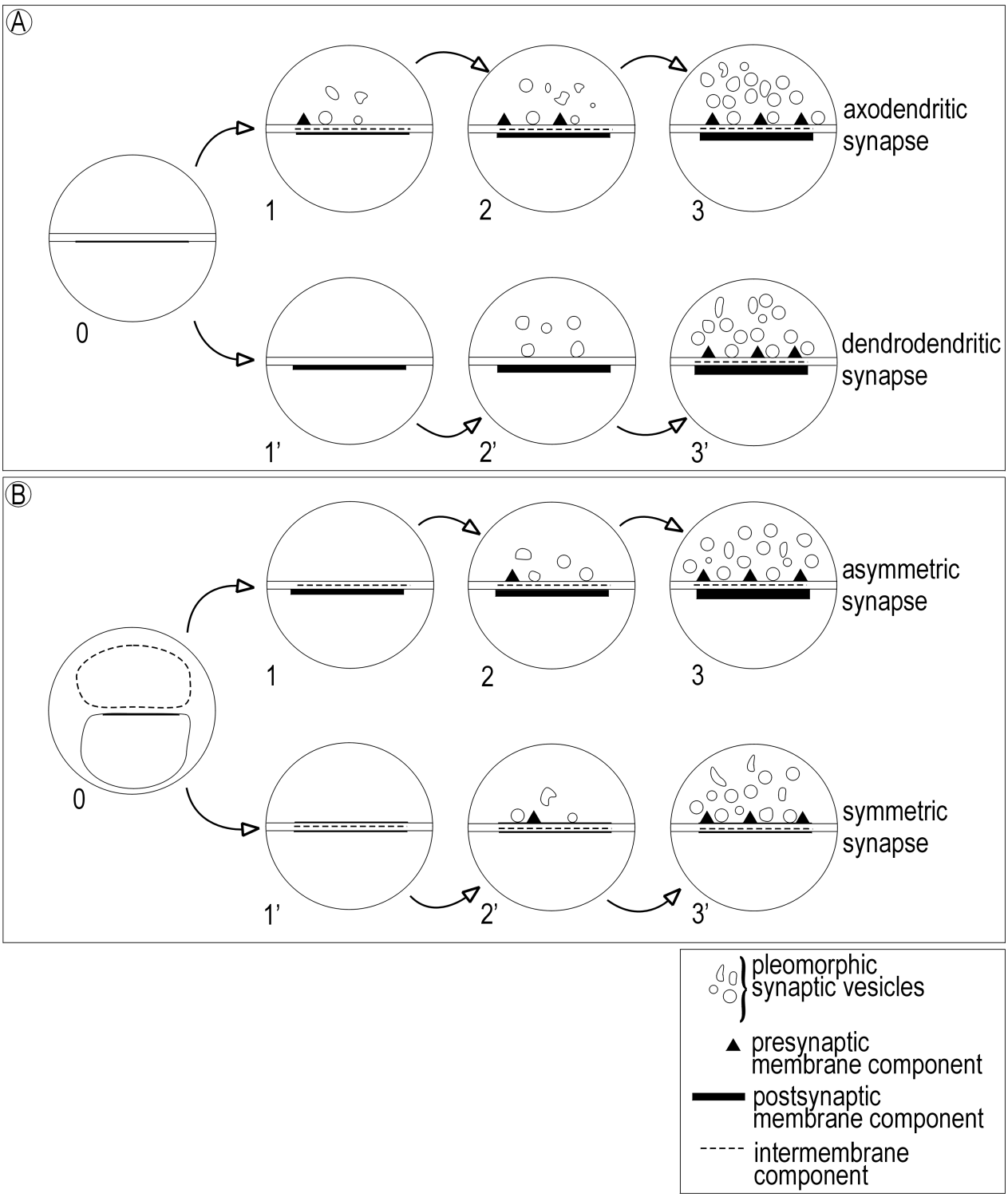

Fig.1) Schematic illustration of proposed sequences of events for synapse formation in (A) the mouse olfactory lobe and (B) the rat visual cortex. **A**) In 0, a hypothetical precursor is illustrated for the two types of synapses found in the developing olfactory lobe. Schemes 1 to 3 are the profiles observed in the formation of axodendritic synapses. Synaptic vesicles are found opposite to a poorly developed postsynaptic density (1). Subsequent development consists of increased postsynaptic density width (2) and further maturation of the presynaptic element (3). Schemes 1' to 3' are the profiles observed in the formation of mitral-to-granule dendrodendritic synapses. Isolated membrane densities resembling postsynaptic densities broaden (1') and then the presynaptic vesicles arrive (2'). This further matures (arrival of presynaptic components and increased numbers of synaptic vesicles) until the adult profile is achieved (3'). (**B**) In 0, the hypothetical precursor of the two types of synapses found in the developing visual cortex is shown. At first a thin postsynaptic density lies opposite to an extracellular space or to an axon and/or dendrite (therefore, doted line). Schemes 1 to 3 are the observed profiles in the formation of asymmetric synapses. The postsynaptic density broadens (1) and then the presynaptic membrane components and synaptic vesicles arrive (2). The synapse further matures until

acquisition of the adult appearance (3). Schemes 1' to 3' depicts the suggested sequence for the profiles observed in the formation of symmetric synapses. Pre- and postsynaptic elements are in contact (1'), presynaptic membrane components and synaptic vesicles arrive without increasing the width of the postsynaptic density (2'). It further matures until acquisition of the adult appearance (3'). Adapted from Hinds and Hinds (1976a) and Blue and Parnavelas (1983a).

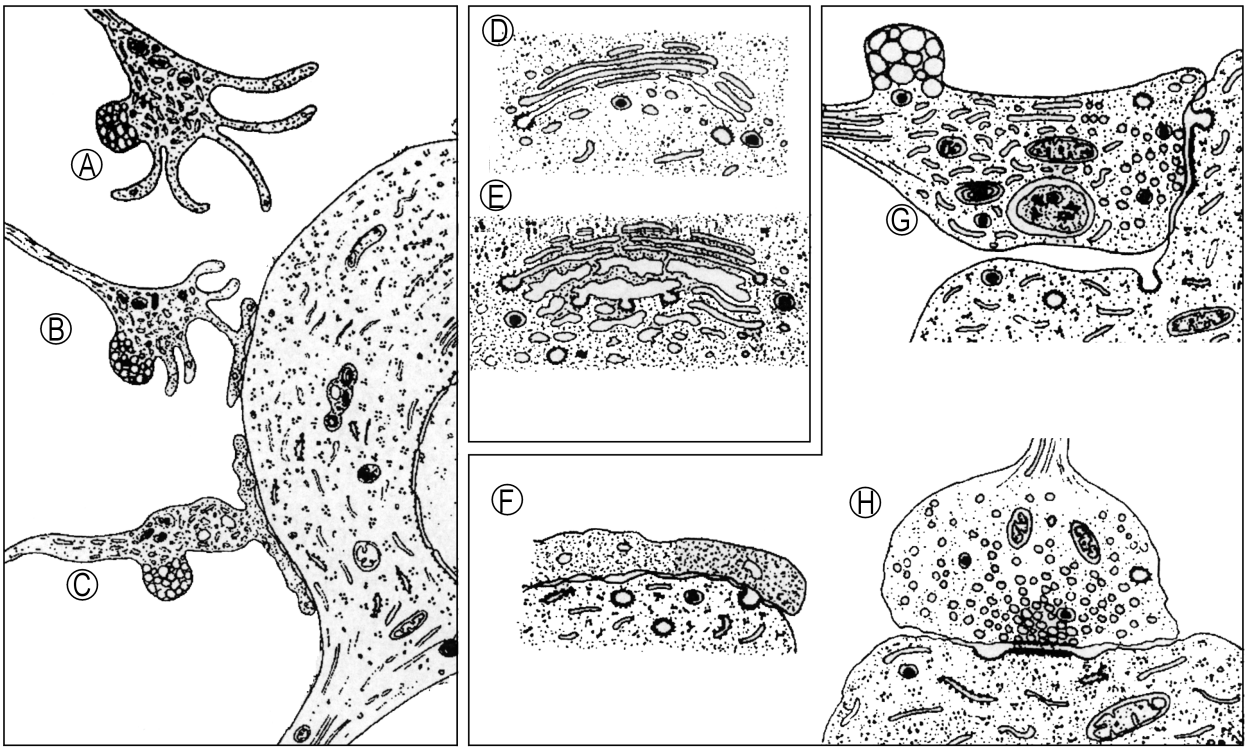

Fig.2) Schematic illustration of *in vitro* synapse formation between the thoracic spinal cord and superior cervical ganglia neurones after 2-3 days in culture. **A**) The somata of a superior cervical ganglia is approached by a spinal cord neuritic growth cone exhibiting filopodia which contain lysosome-like structures and pleomorphic vesicles. **B**) A single filopodium of this growth cone is depicted, having contacted the target neuronal surface. Other filopodia are withdrawing. **C**) Only the highly flattened, contacting process remains, and its surface membrane has developed close contacts with the neuronal plasma membrane at multiple points. **D**) The depiction of a typical Golgi apparatus of a cultured superior cervical ganglia neurone before contact. A few coated vesicles are continuous with cisternae or present in the adjacent cytoplasm together with an occasional large dense core vesicle. **E**) Depiction of changes occurring in the Golgi apparatus of a contacted target neurone. A greater number of coated vesicles are present, some being contiguous with the trans Golgi. **F**) These coated vesicles are believed to migrate to the neuronal surface area where the filopodia contact and fuse with the plasma membrane, thereby contributing to the postsynaptic density. **G**) On the presynaptic side, synaptic and large dense core vesicles appear among the growth cone organelles and cluster in front of the postsynaptic density. **H**) The large amount of membranous reticulum, lysosome-like structures and pleomorphic vesicles, which are typical of the growth cone, are no longer present. Few mitochondria, some reticulum, occasional large dense core vesicles and numerous synaptic vesicles now characterise the ending. Presynaptic dense material gradually appears, some cleft material is present, and the cleft widens itself. Postsynaptic membrane density increases in length as the addition of large coated vesicles continues. Adapted from Rees et al. (1976).

3.1.2) Putative molecules involved in synapse formation

Several systems of cell adhesion molecules are involved in ensuring correct connection between the afferent axon and the target cell (Fig.3).

First, the cadherin/catenin system. Cadherins are calcium-dependent adhesion molecules. They consist of an extracellular domain containing the N-terminal signal peptide and five subdomains, one transmembrane segment and one highly conserved cytoplasmic domain, which interacts with catenin (Fig.3A). The latter attaches to the cytoskeleton (Hatta and Takeichi, 1986; Takeichi, 1988, 1994). Fannon and Colman (1996)

hypothesised that cadherins represent part of the molecular code for the specificity of CNS synapse formation.

Second, the cadherin-like neuronal receptor system. This family is composed of proteins with an extracellular domain containing the N-terminal signal peptide and six subdomains (having high homology to the cadherins), one transmembrane segment and one highly conserved cytoplasmic domain (Fig.3B). The latter has no homology with known cadherins. The cytoplasmic domain interacts with Fyn, a nonreceptor tyrosine kinase, which was shown to have a role in long term potentiation of synapses and spatial learning processes (Grant et al., 1992; Kohmura et al., 1998).

Third, the neurexin/neuroligin system. The synaptic localisation of neurexins has not yet been demonstrated. The mammalian genome has at least three neurexin homologous genes, each of which codes for α and β isoforms (Ushkaryov et al., 1992, 1994). The α-neurexins have a large extracellular, a transmembrane and a highly conserved cytoplasmic domain. The extracellular domain contains the N-terminal signal peptide and three times repeat LNS-EGF-LNS, where LNS is Laminin-Neurexin-Sex hormone binding globulin and EGF is Epidermal Growth Factor. The β-neurexins have a short extracellular, a transmembrane and a highly conserved cytoplasmic domains. The extracellular domain is composed of the N-terminal signal peptide and only one LNS subdomain (Fig.3C). Both isoforms undergo an extensive splicing, resulting in high polymorphism. It was hypothesised that this polymorphism could contribute to the specificity of brain neuronal connections (Ullrich et al., 1995; Obst-Pernber and Redies, 1999). The neuroligins have at least three genes in the mammal genome and consist of an extracellular domain containing the N-terminal signal peptide and a long esterase subdomain, a transmembrane domain and a short cytoplasmic tail. Neuroligins bind specifically to β-neurexins (Song et al., 1999) and the cytoplasmic tail binds to PSD95 (Irie et al., 1997). Even though the neuroligin-1 deficient mouse is viable and fertile (Song et al., 1999), it has been demonstrated *in vitro* that neuroligin-1 triggers the establishment of functional presynaptic structures (Scheiffele et al., 2000).

Other systems (Ephirins, d-glycans, laminins) probably play similar roles in cell-cell interactions and may be involved in the regulation of synapse formation (Brose, 1999; Holder and Klein, 1999; Son et al.,1999).

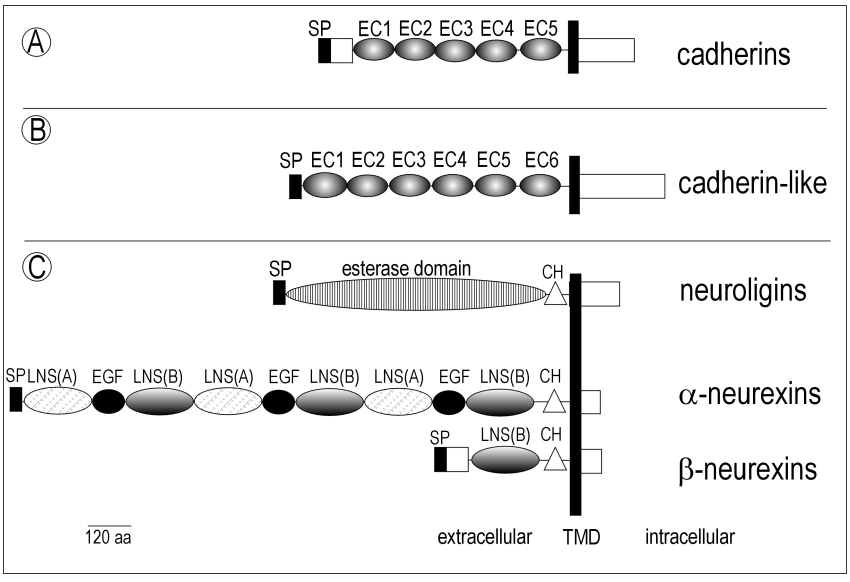

Fig.3) Scheme of cell adhesion molecules. EC: extracellular subdomain; SP: signal peptide; CH: 0-linked carbohydrate attachment sequence; LNS A(B): laminin A (B)/ neurexin/sex hormone binding globulin repeats; EGF: epidermal growth factor; TMD: transmembrane domain (see text). Adapted from Brose (1999).

3.2) Synapse elimination

3.2.1) Morphology of synapse elimination

How CNS synapses are eliminated is a matter of debate. However, it is known that they are eliminated in sensory experience, ageing, after axotomy and hours after a learning experience (Beals, 1976; Wenzel et al., 1980; Holstein et al., 1985; Warton and McCart, 1989; Meyer and Kageyama, 1999).

Axonal retraction with bulb-like shape formation is well characterised in NMJ elimination (Bixby, 1981; Riley, 1981). However, whether CNS synapses disappear by axon retraction or by synapse degradation is not known. There is morphological evidence for axon retraction. After thalamic target cell injury, the afferent axons of the spinal cord neurones form bulb-like swellings (Marty and Peschanski, 1994; Marty et al., 1994). Selective Purkinje cell death induces climbing neurone axons to form bulb-like swellings (Rossi et al., 1993; Rossi and Strata, 1995). However, other types of synapse elimination after axonal injury also exist. 1) Wallerian degeneration. 2) Synaptic stripping. 3) Synaptic autophagy or lysosomal degeneration (for review see Wolff et al., 1995; Aldskogius et al., 1999). Contrasting with the morphological remodelling discussed above, it was also suggested that synapse elimination may occur by disappearance of the vesicles and the active zone, leaving the axonal and dendritic portions intact (Hamori and Somogyi, 1983).

Perhaps all these forms of synapse elimination occur in the CNS. Axonal retraction may occur in case of refinement of synaptic contact. Synaptic stripping, Wallerian degeneration and autophagy may be involved in case of programmed cell death. Simple disappearance of synaptic features, with loss of neither the pre- nor the postsynaptic element, may happen in case of experience-induced synapse elimination.

3.2.2) Molecular events during synapse elimination

The cellular mechanisms underlying synapse elimination are largely unknown. In contrast to synapse formation (see below), synapse elimination seems dependent on synaptic activity. The NMJ elimination is the best characterised synapse elimination process, where the acetylcholine receptor, which is also normally used during cell muscle stimulation, is activated during NMJ elimination. When a small region of the NMJ was irreversibly blocked, the postsynaptic receptors disappeared and the overlying nerve terminals were eliminated. In contrast, when the entire NMJ was similarly silenced, no loss was seen (Balice-Gordon and Lichtman, 1994; Lichtman and Colman, 2000). It is hypothesised that not synaptic activity itself but the pattern of synaptic activity is important for synapse elimination (Busetto et al., 2000). In the cerebellar synapse, synapse elimination may also occur through NMDA receptor activation (Rabacchi et al., 1992; Kakizawa et al., 2000), followed downstream by PKC γ activation (Kano et al., 1995). In the CNS, the role of synaptic activity on synapse elimination is less well known.

4) Synaptic release

The SNARE complex is essential for synaptic vesicle exocytosis (Jahn and Sudhof, 1993; Sudhof, 1995; Bock and Scheller, 1999). This complex consists of a vesicle membrane component, the VAMP/synaptobrevin, as well as the presynaptic plasma membrane components, SNAP-25 and syntaxin. Several adapter proteins regulate this fusion and there may still be new proteins to be discovered. One of those adapter proteins is the munc18-1 protein (Fig.4).

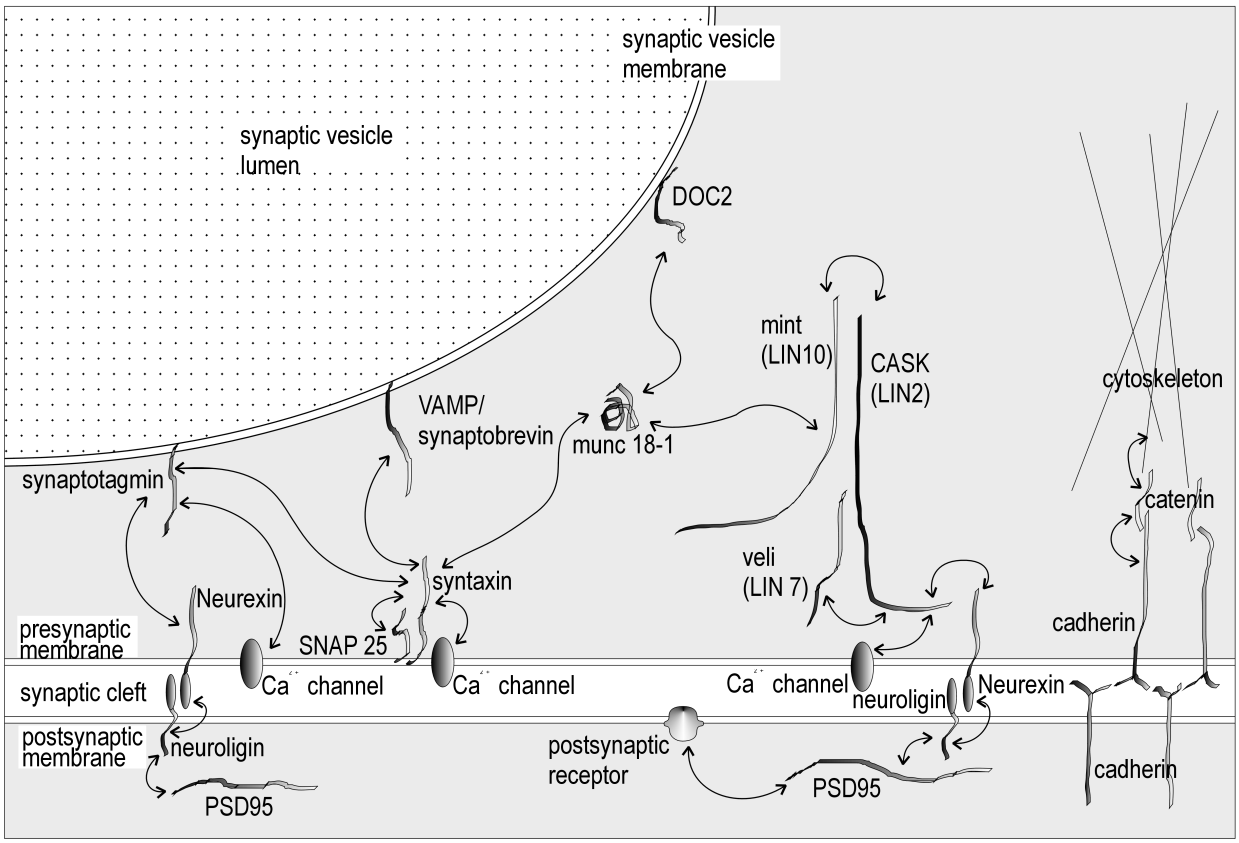

Fig.4) Schematic illustration of some synapse components. Double sense arrows indicate protein-protein interaction. The cell adhesion molecules, such as the cadherin/catenin, neurexin/neuroligin and cadherin-like, are believed to act in the early stages of synaptogenesis. In *C. elegans,* LIN10, LIN2 and LIN7 are responsible for correct targeting of transmembrane proteins. The same sort of role is attributed to their mammal isoforms, MINT, CASK and VELI respectively. Are these mammalian isoforms responsible for targeting synaptic vesicles to the active zone? Synaptic vesicle membrane fuses with the presynaptic element membrane after Ca^{2+} entry into the presynaptic element via voltage sensitive Ca^{2+} channels. Several proteins from the exocytosis machinery bind to those Ca^{2+} channels. Syntaxin/SNAP25/VAMP/synaptobrevin form the SNARE complex, which is essential for secretion. Munc18-1 protein intervenes after docking and before fusion. This protein is necessary for the "fusion-ready" synaptic vesicle pool (via DOC2/MINT interaction?). Its role in fusion may come from the interaction with syntaxin, where munc18-1 changes syntaxin conformation and allows the formation of the SNARE complex.

4.1) Munc18-1 protein

More than 20 years have passed since Novick and Schekman (1979) identified a thermosensitive yeast mutant in which growth and secretion were stopped. In this mutant, secretory vesicles could not fuse and accumulated at the plasma membrane. Working with *C. elegans*, Hosono and colleagues (1987) identified several mutants with uncoordinated (unc) movement, reflecting acetylcholine release deficiency. Among these animals there was the unc18 mutant. Hata and colleagues (1993) identified the unc18 mammalian homologue gene (munc18-1) as binding tightly to syntaxin. Thereafter, the *Drosophila* homologue, rop, which appeared to be essential for secretion was discovered (Harrison et al., 1994; Schulze et al., 1994). Munc18-1 is a 67kDa cytoplasmic brain specific protein (Garcia et al., 1994). It was implicated in synaptic release because of its competition with VAMP/synaptobrevin for binding syntaxin 1 (Pevsner et al., 1994). Munc18-1/syntaxin binding is crucial for the syntaxin availability for core complex formation (Dulubova et al., 1999). Munc18-1 has two isoforms, munc18-2 is found in all tissues (Hata and Sudhof, 1995) and munc18-c is probably specific for insulin responsive tissue (Tellam et al., 1995). Munc18-1 also binds to other proteins like DOC2 (Verhage et al., 1997) and MINT (munc18-1 interacting protein) (Okamoto and

Sudhof, 1997).

5) The role of synaptic activity for synapse formation and elimination

The role of synaptic activity can be studied by 1) impairing action potentials, 2) deleting proteins involved in synaptic vesicle secretion, 3) deleting proteins involved in neurotransmitter biosynthesis, or 4) silencing postsynaptic receptors.

5.1) Action potential blockage

Tetrodotoxin (TTX) is a blocker of voltage-sensitive sodium channels, which prevents action potential propagation and consequently blocks evoked synaptic activity. The Californian newt *Taricha torosa* synthesises and is insensitive to TTX. Grafting an embryonic eye from a TTX-sensitive species, the Mexican axolotl, to the newt blocks action potentials in the retinal ganglion cells of the transplanted eye. Nevertheless such ganglion cells developed normally, grew axons onto the correct tectum target layer and formed functional synapses. The grafted eye developed normal projections even in competition with electrically active axons from a host eye (Harris, 1980). TTX application on cultured rat cortical neurones retarded, but did not prevent neurite outgrowth and synaptogenesis. Furthermore it also retarded the synapse elimination observed in the forth week in culture (Van Huizen et al., 1985). In contrast, the application of the GABA receptor blocker picrotoxin to cultured rat cortical neurones accelerated neurite outgrowth and synaptogenesis and advanced the process of synapse elimination by a full week (Van Huizen et al., 1987). Thus, blockage of action potential (consequently evoked synaptic activity) does not prevent synapse formation or elimination.

5.2) The deletion of protein involved in synaptic vesicle secretion

In addition to evoked synaptic transmission, nerve terminals continuously secrete transmitters with an unknown role by spontaneous fusion of vesicles prior to synapse formation (Xie and Poo, 1986; Sun and Poo, 1987; Gao and Van Den Pol, 2000). The genetic deletion of proteins involved in secretion showed that the secretion process is composed of different steps operated by different proteins (for review see Sudhof, 1995; Cantallops and Cline, 2000). Only few synaptic proteins appear to be essential for secretion munc18 (see section 4.1), syntaxin and munc13.

The absence of syntaxin in *Drosophila* caused subtle morphological defects in the peripheral and central nervous system, affected nonneural secretory events, and entirely abolished neurotransmitter release. From this it was concluded that syntaxin plays a key role in nonneuronal secretion and is essential for evoked neurotransmission (Schulze et al., 1995). The syntaxin deletion in *C. elegans* does not affect embryogenesis, as in *Drosophila,* nevertheless the animals are almost completely paralysed, with only occasional head movements and die soon after hatching. Cholinergic synaptic transmission is completely abolished in the null mutants (Saifee et al., 1998).

Unc13 mutants are reported in mice (Augustin et al., 1999), *C. elegans* (Richmond et al., 1999) and *Drosophila* (Aravamudan et al., 1999) and were not viable. Although all mutants formed synapses, the amount of neurotransmitter released by spontaneous and evoked synaptic activity was greatly reduced.

Thus, although synaptic activity seems to be necessary for animal survival, it is not necessary for synapse

formation. Furthermore, it is plausible that synapses play different roles in pre-natal (via spontaneous synaptic activity) and in adult life (via evoked synaptic activity).

5.3) The deletion of enzymes involved in neurotransmitter biosynthesis

Neurotransmitters are released by synaptic vesicles and trigger an adaptive response by the postsynaptic element. The glutamic acid decarboxylase enzyme synthesises the GABA neurotransmitter. The absence of this enzyme in *C. elegans* does not impair NMJ formation and maintenance (Jin et al., 1999). However in the mouse, in contrast, the acetylcholine neurotransmitter seems to be necessary for cell muscle survival and for establishment and maintenance of the normal anatomical localisation of the synapses (Brandon et al., 2000). Thus, probably in addition to species specificity, the type of neurotransmitter at the synapse, is important for synapse formation, establishment and maintenance.

5.4) Postsynaptic receptor silencing

Postsynaptic receptors are the target of neurotransmitters and trigger postsynaptic element response. It was shown that the absence of the acetylcholine receptor does not impair synapse formation and maintenance at the zebrafish NMJ (Westerfield et al., 1990). However in contrast, postsynaptic receptors seem to play a role in synapse destiny. The postsynaptic acetylcholine receptor is the means by which the mammal NMJ is eliminated (for review see Lichtman and Colman, 2000). In the same way glutamate receptor activation, in a particular period, seems to be critical for synapse elimination during development (Rabacchi et al., 1992; Kakizawa et al., 2000). Furthermore, the transient blockage of glutamate receptors during the period of glutamate receptor hypersensitivity, triggered a wave of apoptotic neurodegeneration (Ikonomidou et al., 1999). Thus in the same way that postsynaptic receptors have a period of sensitivity, it is plausible that synapse destiny depends on a the type of receptor for a species.

6) Thesis outline

This thesis studies the role of synaptic activity in the formation and stability of synapses and in the mouse CNS development. We deleted the munc18-1 gene from the mouse genome and confirmed the hypothesis that munc18-1 protein plays important role in synaptic secretion. Heterozygous animals showed that the munc18-1 protein acts on synaptic vesicle fusion by making the synaptic vesicle “fusion-ready”. Homozygous animals had no synaptic vesicle fusion. In the absence of synaptic secretion, the mouse brain developed normally and subsequently degenerated. By quantifying and characterising mutant synapses, we found that synapses were initially normally formed but could not mature. Anatomical analysis and astrocyte staining revealed that spontaneous synaptic activity is likely to be necessary for co-ordinating cell differentiation and survival.

Chapter 2 describes the synaptic role of munc18-1 protein and shows that synaptic transmission is highly dependent on munc18-1 levels. Chapter 3 describes the consequences of no synaptic transmission on brain development and shows that the brain develops normally but subsequently degenerates. Chapter 4 describes synapse formation without synaptic vesicle fusion and shows that synapses are formed but do not mature. Chapter 5 describes the consequences of no synaptic transmission on cell differentiation and shows that some neurones need synaptic transmission to differentiate while others do not, and gliogenesis occurs prematurely.

chapter 2

Munc18-1 is essential for synaptic secretion by making synaptic vesicles fusion-ready

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ABSTRACT

Munc18-1 is a neuron-specific protein implicated in regulated exocytosis. We analysed its synaptic function by reducing and deleting its expression in mice. Munc18-1 deletion abolished synaptic transmission, although synapses containing (docked) synaptic vesicles and functional postsynaptic receptors were present. Six synaptic proteins exhibited reduced levels: syntaxin-1A and B, DOC2A and B, synapsin-1 and rabphilin-3A, but syntaxin-immunoreactivity was observed in synaptic areas. Heterozygous mice only had 50% reduced munc18-1 levels and showed reduced spontaneous vesicle-fusion, a reduced number of vesicles secreted per stimulus, a reduced response to hypertonic solutions and early fatigue at high frequency stimulation. Hence, reduced munc18-1 expression decreased the availability of synaptic vesicles. We conclude that munc18-1 is an essential factor in synaptic secretion by making synaptic vesicles 'fusion-ready'.

INTRODUCTION

Neurotransmitter secretion from synaptic vesicles in the mammalian brain depends on a complex interaction of many genes. Together with vesicle trafficking between Golgi and plasma membrane in yeast, the life-cycle of synaptic vesicles is one of the most intensively studied examples of intracellular vesicle transport (see for reviews Bennett and Scheller, 1994a, b; O'Connor et al., 1994; Sudhof, 1995). In particular the central role of the trimeric complex, consisting of syntaxin, synaptobrevin/VAMP and SNAP25 in the fusion reaction is now firmly established and serves a model for a large number of other fusion reactions in different cellular compartments in different species (Hanson et al., 1997a, b; Sutton et al., 1998; Weber et al., 1998; Chen,YA et al., 1999). Several proteins have been implicated in the assembly and regulation of these so-called SNARE-complexes. A major controversy exists on the role of SEC1/munc18-like (SM-)proteins. Members of this family are essential for secretion in yeast (Novick et al., 1981; Aalto et al., 1991), in *C. elegans* (Hosono et al., 1992), in *Drosophila* (Harrison et al., 1994) and in mice (Verhage et al., 2000). This suggests that the genes of the SEC1 family are responsible for an essential step in secretion. At present, the nature of this step is unknown, but it appears to be similar between species since in *C. elegans*, the defects caused by unc-18 deletion can be rescued by a mammalian isoform (Gengyo-Ando et al., 1996).

The munc18-1 protein interacts tightly with the plasma membrane protein syntaxin-1 (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994b) and that this interaction is not compatible with the association of SNAP-25 and synaptobrevin/VAMP to syntaxin (Pevsner et al., 1994a). Similarly, incompatibility of the interaction of SM-proteins and synaptobrevin/VAMP with syntaxin proteins have been observed in other systems, involving munc18-2 (Riento et al., 1998) and munc18c (Tellam et al., 1997). Based on these observations, it has been suggested that at least one aspect of munc18 function is to (negatively) regulate syntaxin availability, thereby preventing formation of SNARE-complexes (Pevsner et al., 1994a). However, transfection experiments failed to confirm this suggestion in chromaffin and PC-12 cells (Graham et al., 1997). Meanwhile, two additional protein families have been observed to interact with munc18-1, i.e., DOC2A/B (Verhage et al., 1997) and X11/MINT-proteins (Okamoto and Sudhof, 1997). These interactions may represent additional cascades regulated by the munc18-1 gene.

We have recently shown that munc18 protein and munc18 dependent transmission are not essential for synapse formation in the developing brain (Verhage et al., 2000). Here we have analysed the synaptic function of the munc18-1 gene by exploiting homozygous and heterozygous mice. Deletion of a single allele of the gene resulted in a gene-dose effect, which allowed us to analyse neuronal function both in the absence of munc18-1 (homozygous mutants), and upon a two-fold reduction of its expression level (heterozygous mutants). Here we show that neuronal function, is highly dependent on munc18-1 levels: a selective defect in synaptic transmission was observed upon lowering munc18-1 expression, whereas transmission was completely abolished in the absence of munc18-1.

METHODS

Homologous recombination

A murine genomic library in λ-FIX (Stratagene) was screened with a 0.36Kb NcoI/XhoI fragment from the 5'

end of the rat munc18-1 cDNA encoding residues 1-120. Four of 32 positive clones were analyzed in detail and contained four exons encoding residues 14 to 108 (Fig.1A). A tragetting vector was constructed from two overlapping l-clones (#1 and 11) in which all four exons were replaced by a neomycin resistance gene for positive selection. The neomycin gene was flanked by an 11.5Kb long arm and a 1.4Kb short arm which in turn was flanked by two copes of the Herpes simplex thymidine kinase gene for negative selection. ES cells ("G-cells", kind gift of Dr. J. Herz, Dallas) were transfected and selected as described (Rosahl et al., 1995) and analyzed for homologous recombination using PCR with oligonucleotide A: outside sense primer CGGTACTTGGGGATTGAACCCAGGC; B: Neo antisense primer to detect the mutant allele GGATGCGGTGGGCTCTATGGCTTCTGA; and C: inside antisense primer to detect wild type allele, AAAGGAACGGGGTGGAGGGAGAGA. Putative homologously recombined clones were confirmed by Southern blotting with outside probes (indicated by 1 and 2 in Fig.1A). Two positive ES cell clones were injected into mouse blastocysts, generating highly chimeric mice that transmitted the gene through the germline. Mice were bred as heterozygotes using standard mouse husbandry. The genotype of embryos from heterozygote matings was analysed before birth and exhibited a Mendelian distribution of allele combinations (Fig.1B and data not shown). To monitor the heart beat in mouse embryos, two subcutaneous electrodes were extended through the uterus of the anaesthetised mother, and the genotype of the pups was analysed afterwards.

Histology

Mouse embryos were collected after timed matings at days embryonic 16 (E16) and E18. Embryos were immersed in 71% saturated picric acid, 23.8% formalin, 0.5% acetic acid and 4.7% water for 2 days. After dehydration in a graded series of alcohol followed by xylene, tissues were embedded in paraffin. Whole brain sections of 5μm were cut, attached to gelatine-coated slides, and stained with hematoxylin/eosin or cresyl violet.

Neocortex electrophysiology

Spontaneous IPSCs were recorded at 33°C as described previously (Brussaard et al., 1997). In short: normal ACSF was used containing 125mM NaCl, 25mM $NaHCO_3$, 3mM KCl, 1.2mM NaH_2PO_4, 2.4mM $CaCl_2$, 1.3mM $MgSO_4.7H_20$, 10mM D-glucose (304mOsm, carboxygenated in 5% CO_2/95% O_2, pH7.4). The pipette solution contained 135mM CsCl, 1mM $CaCl_2$, 10mM EGTA, 10mM Hepes, 2mM MgATP and 0.1mM GTP (acid free), adjusted to pH7.2 using CsOH (296mOsm). Bicuculline was obtained from RBI (Natick, MA). The spontaneous IPSC recordings were stored on DAT recording tapes and analysed off-line using the J. Dempster CDR and WCP software (Univ. Strathclyde, Glasgow, UK) at a sampling rate of 10kHz. All ecordings were obtained at a holding potential of -70mV.

Electron microscopy

Brains of E16 mouse embryos were dissected and immersed in 1% paraformaldehyde and 2% glutaraldehyde in 0.05M sodium cacodylate buffer (pH7.4) for 2 days at 4°C, postfixed in 1% OsO_4 in 0.1M cacodylate buffer (pH7.4), dehydrated, and embedded in EPON (Pou 812). Ultrathin (±90nm) sections were mounted on non-coated copper grids and contrasted with uranyl acetate/lead citrate. Morphometric analysis was performed on 3 random sections (approximately 1700μm^2 each) per animal (n=4) from the marginal zone of the neocortex, close to the midline of the brain and above the rostral boundary of the hippocampus.

In these analyses, synapses were defined as structures containing one or more vesicles of about 50nm and an active zone (electron dense membranes). In each experimental group at least 50 of such structures were analysed. The size of the synapses and the length of the active zone were evaluated in the same sections and quantified using a calibrated scale in the electron microscope. Docked vesicles were defined as vesicles morphologically attached to the active zone and opposed to a postsynaptic density.

Protein analysis.

Mouse brains were obtained at E18 by caesarean section from heterozygous matings. Brains were solubilized by sonication on ice (4 x 5 sec) in 10mM Hepes/KOH (pH7.4) containing 1mM EDTA and protease inhibitors (PMSF, 0.1g/l; leupeptin, pepstatin and aprotinin, 1mg/l each). After sonication, Triton-X-100 and NaCl were added (final concentrations: 1% (v/v) and 150mM, respectively). The homogenates were rotated for 1h at 4°C and centrifugated at 100,000g for 1h. The solubilized proteins in the supernatant were quantified using Bradford's protein assay and equal amounts were subjected to 8-15% SDS-PAGE. Typically, each blot contained 3 protein concentrations, 1μg, 10μg and 100μg of each of the 3 genotypes. For abundant proteins 1, 5 and 10μg were loaded. Proteins were transferred to nitrocellulose membranes (Hybond-C extra, Amersham. UK) with constant current (3mA/cm^2). Blots were preblocked using 5% (w/v) powdered milk and 5% (v/v) goat serum, and immunostained with various antisera (see below). Data were corrected for local blot efficiency using internal standards, i.e., ubiquitous proteins in neurones as well as glia, expressed from early stages of development onwards. Depending on the size of the protein investigated, the following internal standards were used: calmodulin (17kDa), GDI (54kDa) and hexokinase (96kDa).

The following monoclonal antibodies from Synaptic Systems, Göttingen, Germany (except munc18-1: Transduction Labs, Lexington) were used (code names given in brackets when applicable): synaptotagmin I (Cl41.1); synaptobrevin II (Cl69.1); rab3A/C (Cl42.1); NMDA-R1 receptor (Cl54.1). The following polyclonal antibodies were used: munc18 (J370); SNAP-25 (I733); syntaxin 1 (I378); SCAMP (R806); synaptophysin 1 (P611); synaptoporin/synaptophysin 2 (Y941), synaptogyrin (ap29); rabphilin-3A (I374); synapsins (E028); DOC2A/B (I734 and N321); NSF (J372); rab4 (10503); B50/GAP-43 (ab8921), calbindin-28K (SWant, Bellinzona); VCP (K331); MINT (P730); rabaptin (Q699).

Immunohistochemistry

Sections (see histology) were rehydrated, incubated with 0.1% Froggy® detergent (15-30% anionogenic, <5% ionogenic and amphoteric surfactants; Werner and Mertz, Waterloo, Belgium) in 50mM TBS pH7.5, microwaved (4x 5min) and washed 3 times in TBS. Endogenous peroxidase was blocked with 0.6% H_2O_2 in 100% methanol for 30min at RT. Sections were washed again 3x in TBS and incubated for 1h at RT in 3% normal goat serum (NGS), 50mM TBS, 1% BSA and 0.1% Triton-X-100. For staining with monoclonal antibodies, NGS was replaced by 1% goat anti-mouse serum (Sternberger Monoclonals). Sections were incubated with primary antibodies overnight at RT, subsequently with biotinylated secondary antibody for 1h at RT with peroxidase-labelled streptavidin-biotin complex, and finally with 3',3'-diaminobenzidine in 50mM Tris/HCl pH7,6. Antibodies used were 9527 (GAP-43), 1:5000 and E028 (synapsins), 1:1500. Control experiments omitting primary and secondary antibodies confirmed specificity of the stainings.

RESULTS

Null mutants are born alive, but are paralysed and die shortly after birth

We manipulated munc18-1 expression in mice by homologous recombination in embryonic stem cells (two independent lines; Verhage et al., 2000; Fig.1A). Heterozygous mutant mice were viable and fertile, but homozygous mutant mice were paralysed at birth, died within 1h and showed no synaptic transmission (Verhage et al., 2000). The general appearance of the mutant pups was unremarkable (Fig.1B) and a steady heartbeat (Fig.1C) was monitored in the mutants *in utero* after insertion of bipolar electrodes through the uterus wall in the anaesthetised mother. Visual inspection after dissection/surgery revealed that heart activity persisted for some time after birth, indicating that the mutant pups were born alive. No gross abnormalities in external or internal organs were noticed, except for an expanded, urine filled bladder (not shown). Closer inspection under the microscope revealed that the null-mutants also had thinner muscles, which may be a consequence of a generalised muscle paralysis. The null mutants exhibited an atypical neuronal degeneration, affecting primarily the basal areas of the brain. The neocortex was completely assembled, segregated into layers and fibre pathways were targeted correctly and synapses were formed (Verhage et al., 2000). Concomitantly, a significant decrease in total brain protein content was observed in the homozygous mutants at birth: 76 ± 7% of wild type littermates, $p<0.05$. Late developing brain areas such as the neocortex, were indistinguishable between homozygous mutants and wild type littermates at birth. Therefore, the neocortex was used to investigate the synaptic structure and function in the munc18-1 null mutants and to exclude potential influences of neuronal degeneration.

Null mutant neocortex has a normal cell-layering and synapse-morphology, but no transmission

The overall structure of the neocortex, its layering and the density of neurones in the cortical plate were similar in null-mutant and control brains (Fig.2A-C). Whole cell voltage clamp recordings in neocortical slices from control pups at E18 revealed frequent, spontaneous postsynaptic currents (5.4 min^{-1} with a peak amplitude of 35.6 + 9.4 pA; n=3 animals, n=5 recordings). In contrast, in slices from munc18 deficient littermates no spontaneous synaptic current event was detected throughout a total recording period of 30.8 min (Fig.2D; n=3 animals, n=9 recordings). However application of GABA, the predominant neurotransmitter in this area at this developmental stage (Ben-Ari et al., 1997), induced a typical, slowly decaying bicuculline-sensitive whole cell current. Using electron microscopy, we analysed the neocortical marginal zone of munc18-1 deficient mice and found structures with all morphological aspects of a synapse, i.e., a presynaptic element with clustered and sometimes docked synaptic vesicles, and a juxtaposed postsynaptic element and electron dense membrane (active zone) (Fig.2E and data not shown). The ultrastructure of the neocortex in homozygous mutants was indistinguishable from wild types with respect to axon, dendrite and soma morphology (Fig.2 and data not shown). We observed no degeneration, confirming the conclusions from the light microscopical data in Fig.2. The synapses of munc18-1 deficient mice were similar in appearance to those observed in control littermates (see chapter 4). Since the homozygous mutants die shortly after birth, we cannot analyse the morphology of mature (postnatal) synapses. However, we concluded that, at least during initial synaptogenesis, munc18-1 deletion does not affect synapse morphology. Hence, munc18-1 is absolutely essential for secretion, but not for the formation of synapses, the targeting of synaptic vesicles to the nerve terminal, the presynaptic accumulation of these vesicles and their docking at the site of release. Deletion of munc18-1 expression

does not affect the morphology of developing neocortical synapses, suggesting that it blocks a biochemical rather than a morphological aspect of secretion, notably downstream of vesicle docking.

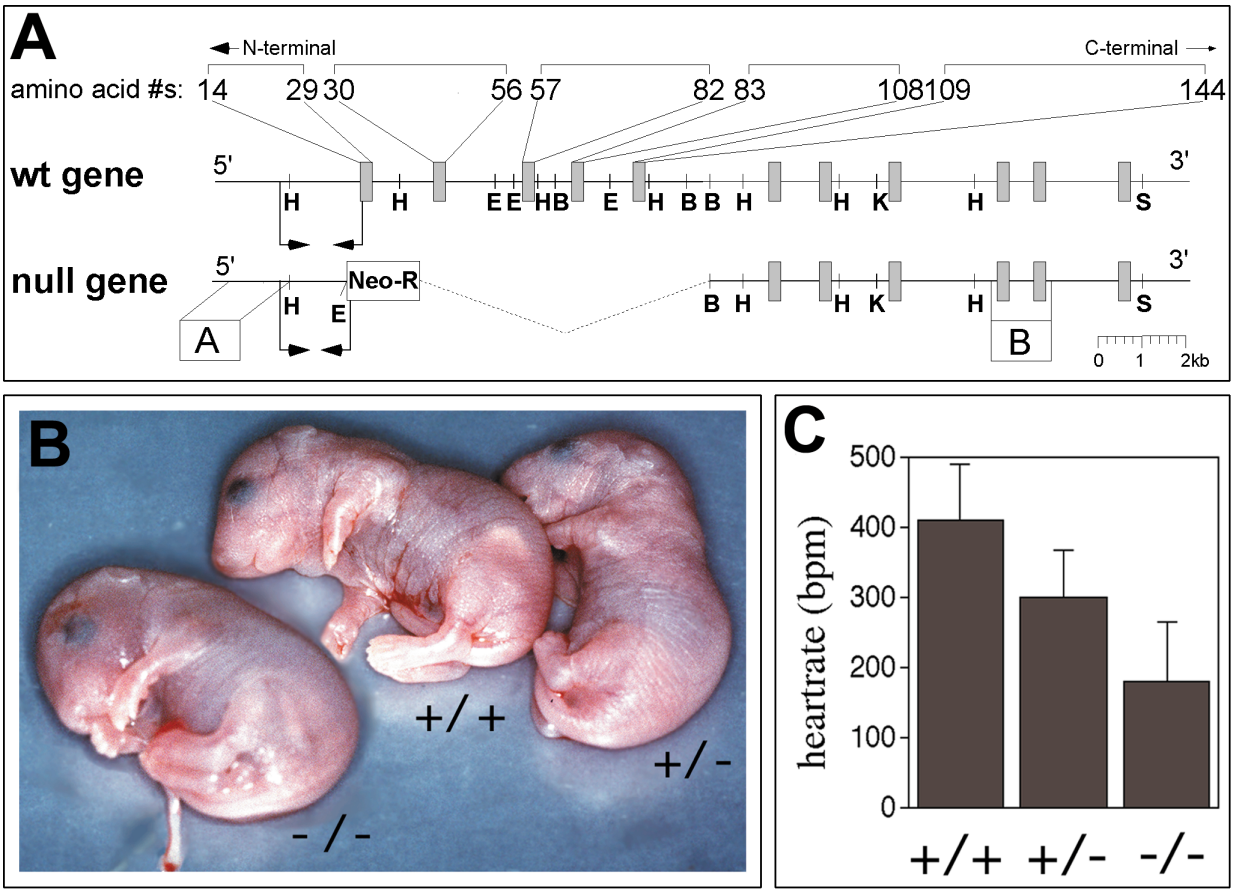

Fig.1) Generation of the munc18-1 knockout mice. **A**) Structure of the 5' end of the wild type munc18-1 gene (wt gene) and the mutant munc18-1 gene after homologous recombination (null gene). Exons are represented by gray boxes, with the numbers of the encoded amino acid residues shown above the wt gene. Homologous recombination replaces five exons with a neomycin resistance gene (NEO-R). Pairs of arrows indicate the locations of the PCR primers used for genotyping and white boxes labelled A and B indicate the probes used for Southern blot analysis. Single letters mark the position of restriction enzyme sites: H, HindIII; E, EcoRI, B, BamHI; K, KpnI; and S, SmaI. **B**) General appearance of the pups of the 3 genotypes (as indicated) from a single litter. Pups were obtained after caesarean section at E18. **C**) Analysis of the heart rate of the pups at E18 by insertion of bipolar electrodes through the uterus wall of the anaesthetised mother. Bars indicate averages of 3-4 recordings ± SEMs.

Null mutants have reduced levels of six proteins, but these proteins can be targeted to the synapse

To analyse the potential biochemical changes that form the basis for the inability to secrete transmitter in munc18-1 deficient mice, we compared the levels of 27 proteins between mutants and wild type littermates. As mentioned above, the brains of the mutants had 25% less wet-weight. Consequently, the total protein content in a whole brain homogenate was also lower in the mutant mice and therefore all protein levels were standardised using 3 standards (ubiquitous proteins with general functions: hexokinase, calmodulin and GTP-dissociation inhibitor (GDI)). We found that most proteins were present at similar relative concentrations in wild type, heterozygous, and homozygous mutant brains (Fig.3). Intrinsic membrane proteins of synaptic vesicles (synaptotagmin, synaptophysins, synaptobrevin, SCAMP) were unchanged, in line with the electron microscopic observation that the mutant synapses contain synaptic vesicles. In addition, presynaptic and postsynaptic plasma membrane proteins (GAP-43/B50 and NMDA-R1 receptor respectively) were present in relatively similar amounts in all genotypes, as were the cytosolic proteins

involved in membrane traffic (SNAP-25, NSF, rab3, rab4, VCP). Two types of proteins were lower in the mutants: (A) Four known munc18-1 interacting proteins. Syntaxins 1A and 1B were decreased by approximately 70%, and DOC2A and 2B by almost 40%. This confirms *in vivo* what was previously suggested by *in vitro* data, i.e. that munc18-1 interacts with syntaxin 1 and DOC2. (B) Two peripheral membrane proteins of synaptic vesicles. Synapsin-1 and rabphilin-3A were decreased by approximately 50%. Thus of 27 proteins studied, only six exhibited changes, four of which are known munc18-1 interacting proteins. Hence, in spite of a drastic interference with synaptic function, no major rearrangements of synaptic protein composition were observed, but apparently, a small selection of synaptic proteins depends directly or indirectly on munc18-1 for their stability.

Immunocytochemical staining of the mutant brains with antibodies for two of the down regulated proteins synapsin-1 and syntaxin-1, revealed abundant staining in fibres such as the commissural fibres, and in synaptic regions, such as the marginal zone of the neocortex (Fig.4). Hence, munc18 deficiency does not prevent export and targeting of these proteins from their site of synthesis to the nerve terminals. This suggests that the lower levels of these proteins is not a consequence of a defect in their processing, sorting and/or targeting induced by the absence of munc18-1.

Heterozygous mutants have a reduced pool of fusion-ready vesicles

Mice lacking a single allele of the munc18-1 gene showed an approximately 50% reduced munc18-1 expression at the protein level, but no similar reduction in the levels of any of its known binding partners or 22 other proteins (see Fig.3). These mice were viable and fertile and showed no signs of neuronal degeneration throughout the brain (data not shown). Similar protein levels were found in adult mice, and the munc18-1 reduction was similar in different brain areas (data not shown). After 8 weeks, levels of syntaxin tended to decrease, although this decrease was never more than 20% (n=5). Because munc18-1 is the only protein with lowered expression in young heterozygous mice, these mice allow a more direct analysis of the molecular function of munc18-1.

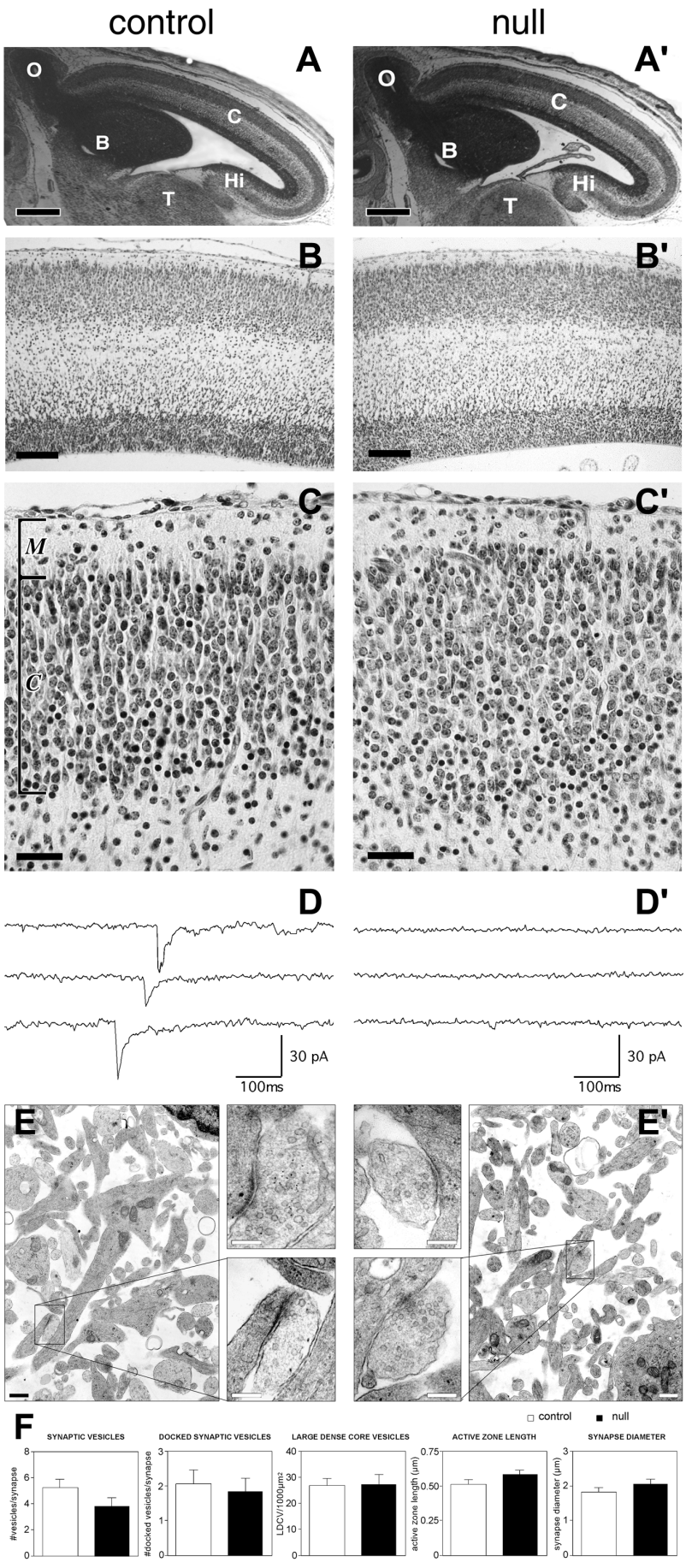

Fig.2) Structure of munc18-1 deficient mouse forebrain, neocortex and neocortical synapses, and their transmission. **A**) Sagittal sections of hemotoxylin-eosin (HE) stained forebrain from wild type (A) and knock-out mouse embryos (A') at E18. Major brain areas are indicated: O: olfactory bulb; C: cortex; B: basal ganglia; Hi: Hippocampal formation; T: thalamus. Bar: 500µm. **B**) HE-stained sections of the neocortex Bars: 100µm **C**) HE-stained sections of the marginal zone (M) and cortical plate (C) Bar: 25µm **D**) Whole cell voltage clamp recordings of spontaneous synaptic currents in the cortical plate region of acute neocortex slice preparations at E18. Holding potential was -70mV at 33°C. Amplitude distribution of synaptic current events with a log-normal function fitted to the data revealed a maximal event amplitude of 40 ± 2pA (n > 200 events) from the control recording and a frequency of 0.41 ± 0.10Hz. We did not detect a single spontaneous synaptic event in 9 recordings made in mutant slices (n=3 animals, total recording time of 1849s). Ionthophoresis of GABA (1mM, 200-400nA, 10-20 pulses of 100ms duration at 4Hz) induced a slowly decaying whole cell current that was sensitive to the $GABA_A$ receptor antagonist bicuculline (20µM in bath) and occasional spontaneous action potentials in the cell attached mode at 33°C were observed in the mutants as well as voltage gated inward ion current responses in whole cell mode (see also chapter 3). This indicates that the sampled cells were neurons, not glia cells and that these neurons exhibited otherwise normal electrophysiological properties in the absence of munc18-1. In addition, series resistance and whole cell capacitance were similar in control and mutant slices (R_S=14±7MΩ, C=6.7±3.1pF, n=5; and R_S=13.7±8.5MΩ, C=6.7±3.8pF, n=9, respectively). This indica-tes that the total cell surface area, i.e. the cell size and arborization, were similar between mutants and controls. **E**) Representative over-views and close-ups of nerve terminals in the marginal zone of the neocortex at E16 of control and mutant mice. Nerve terminals have a normal appearance, revealing active zone/postsynaptic density complexes and clustered and docked synaptic vesicles. Bar: 1µm (overview panels) and 200nm (close-up). **F**) Morphometric quantification of the synaptic organization in the same samples. Data indicate averages ± SEM, n=4 animals in each group, 3 sections per animal (more than 5000μm^2)

We performed intracellular recordings of endplate potentials (EPPs) and miniature EPPs (MEPPs) from the diaphragm neuromuscular junction, because this allows the analysis of synaptic function at the level of a single synapse. As in the neocortex, the evoked synaptic transmission and spontaneous synaptic events were completely absent in the neuromuscular junction of null mutant mice (Fig.5). In adult heterozygous mice we found a 25% reduction in the frequency of spontaneous acetylcholine release events (MEPP frequency). No differences were observed in the resting membrane potential, quantal size (MEPP amplitude) and upward or downward slopes of the events (data not shown).

We characterised the presynaptic effects of lowered munc18-1 expression further in the adult diaphragm. Concomitant to the reduction in MEPP-frequency, we observed a reduction in 0.3 Hz-evoked acetylcholine release (quantal content; Fig.6A). Since neuromuscular transmission has a substantial 'safety factor' to reliably transmit impulses, the reduction in evoked release did not lead to paralysis of the diaphragm (each nerve impulse resulted in muscle contraction when tested for in the period prior to μ-conotoxin treatment to enable EPP measurements). No changes were observed in evoked response latency (data not shown). The lower level of quantal acetylcholine release suggests a reduction of either the number of releasable vesicles (n) or the probability of release (p) of a vesicle. Using the method of (Miyamoto, 1975), it appeared that p was unchanged at heterozygous endplates while n was reduced by 21% (Fig.6). To obtain further evidence for a reduced number of releasable vesicles we applied hypertonic medium (0.5M sucrose) to probe for the fusion-ready pool (Hubbard et al., 1968; Stevens and Tsujimoto, 1995). The sucrose-induced high MEPP frequency was 31% lower at heterozygous munc18-1 endplates (Fig.6A/B), almost proportional to the reduction of munc18-1 protein. Hence, reduction of munc18-1 expression leads to reduction of the pool of releasable vesicles. Finally, a reduced number of releasable vesicles was also suggested from repetitive nerve stimulation protocols. At 40Hz nerve stimulation, a rundown of EPP amplitude was observed in normal animals to a steady level of approximately 78% of the first EPP (Fig.6C). This is determined mainly by the rate of replenishment of the releasable vesicle pool. At heterozygous endplates, the rundown was significantly greater (to 67% of the first EPP, $p < 0.01$). Similar differences between heterozygotes and wild types in the rundown of the cellular response were observed in EPSP analysis of the CA1 area of the hippocampus (data not shown). Hence, the reduced availability of synaptic vesicles for secretion upon reducing munc18-1 expression is a general phenomenon, both in the peripheral nervous system and the CNS.

Statistical analysis of the synaptic ultrastructure of heterozygous mutants and wild type litter mates revealed that reduction of munc18-1 expression does not affect the distribution of synaptic vesicles in the neuromuscular junction. Heterozygous animals contain neuromuscular synapses packed with numerous synaptic vesicles, which were also docked at the membrane, as in normal neuromuscular synapses (Fig.7). This is in line with the data from homozygous mice indicating that manipulation of munc18-1 expression does not affect the organisation of the nerve terminal, but does affect a biochemical step at the site of release.

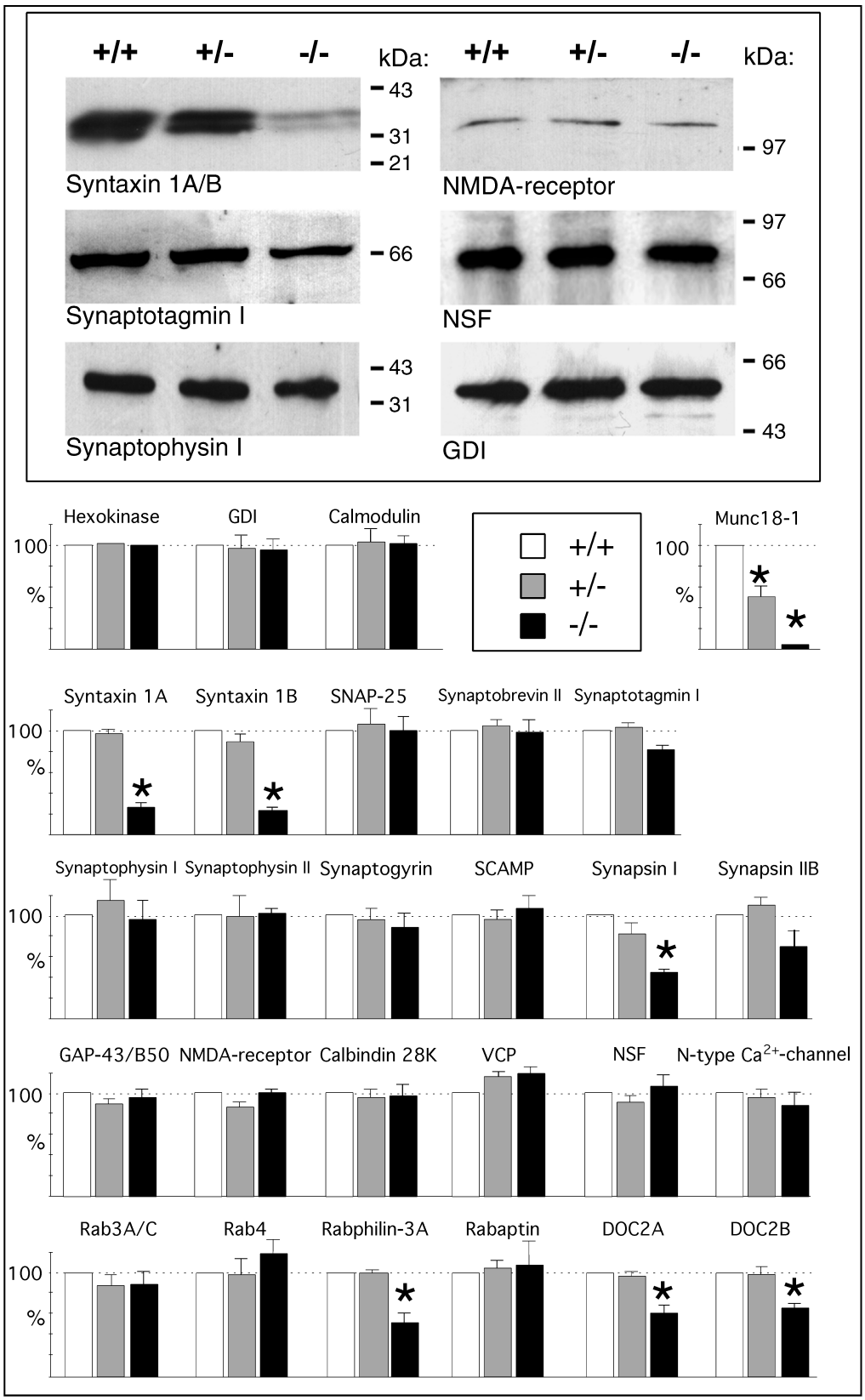

Fig.3) Quantification of protein levels in wild type and mutant mice. Graphs show the quantification of immunoblot analysis of brain homogenates from E18 mouse pups using specific antisera to the proteins shown and phosphoimager detection of iodinated secondary antibodies. Immunoblots were loaded with 3 different amounts of brain protein from littermates of all genotypes, and signals were normalised for the hexokinase and/or GDI le-vel as a general marker. On a per protein basis, hexokinase levels were 84.0 ± 3% (n=18) of wild type in the homozygous mutants. Data are averages ± SEM; n=4 to 8. Proteins with a significant decrease in knockout mice are indicated by an asterisk ($p < 0.05$-0.001).

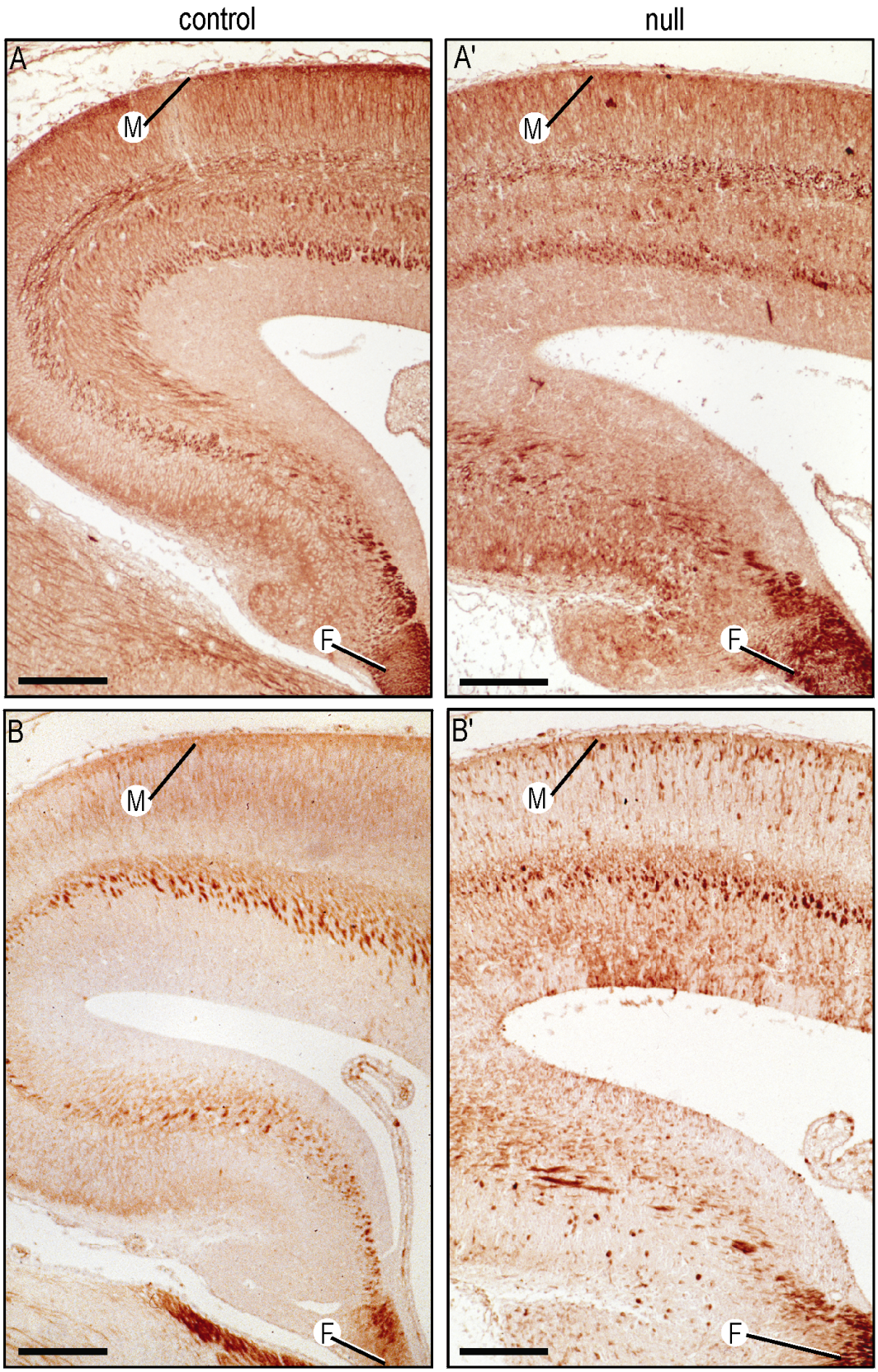

Fig.4) Immunocytochemical localization of synapsin and syntaxin in the cortex of wild type and homozygous mutant littermates. (**A, A'**) synapsin (**B, B'**) syntaxin. M: marginal zone (synaptic layer), F: fimbria fornix.
Bar: 200μm.

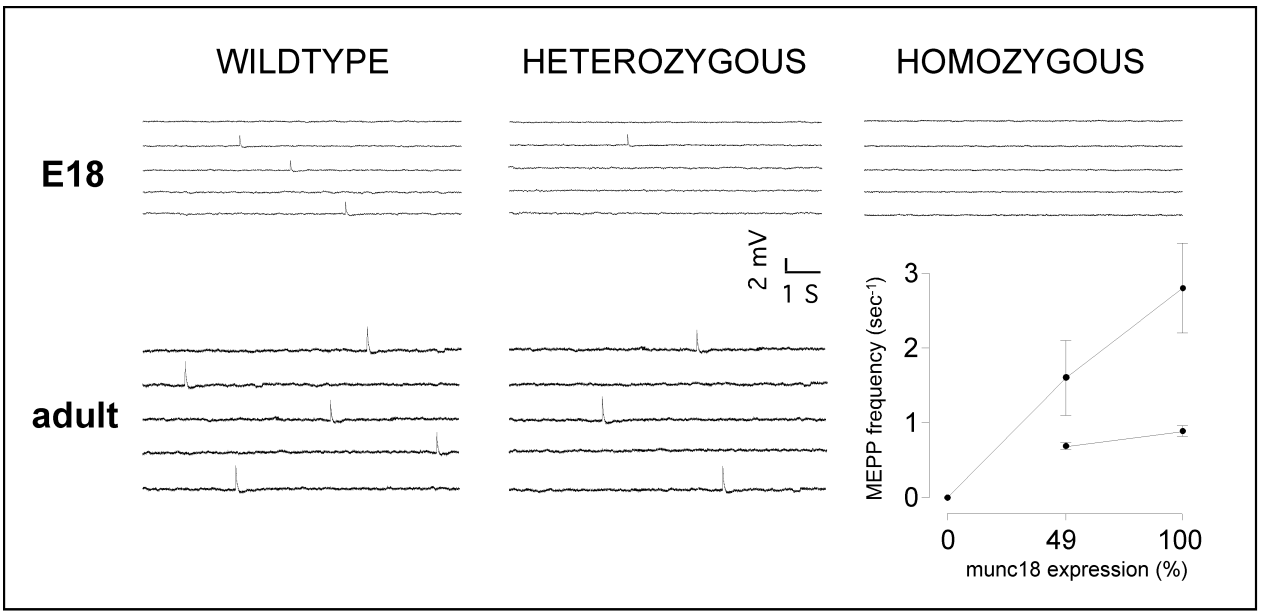

Fig.5) Analysis of spontaneous miniature endplate potentials in nerve-muscle preparations from control and null mutants mice. Spontaneous miniature endplate potential (MEPP) frequency was recorded for at least 13min per animal (n=4-5) in adult and late embryonic (E18) stages. Representative traces are shown, reflecting the relative abundance of the events in the three genotypes. Average frequencies are plotted against the munc18-1 protein levels (see text).

DISCUSSION

Deletion of the munc18-1 gene in mice resulted in a paralysed organism that died shortly after birth. These mice had heart activity, but no spontaneous or evoked secretion throughout their nervous systems, although their synapses contained (docked) synaptic vesicles. The levels of six synaptic proteins, including four munc18-1 binding partners, were lower in the absence of munc18-1. A two fold reduction in munc18-1 expression by deleting a single allele of the munc18-1 gene, resulted in a reduced frequency of spontaneous vesicle-fusion, a reduced number of vesicles secreted per stimulus, a reduced response to hypertonic shock, and an increased run-down of responses during high frequency stimulation.

Munc18-1, the mammalian, neurone-specific member of the SEC1 family, has an essential function in the tissue where it is expressed, similar to munc18-1 isoforms in non-mammalian systems (Novick et al., 1981; Hosono et al., 1992; Harrison et al., 1994). In fact, the phenotype of munc18-1 deficient mice is more severe than any published deletion of genes implicated in secretion (summarised by Fernandez-Chacon and Sudhof, 1999). The munc18-1 deficient mouse is the first gene deletion that results in a complete and widespread loss of all transmitter secretion, both spontaneous and evoked. Apparently, two other munc18 genes in mice can not rescue this deletion. We conclude that munc18-1 is a general, essential gene for neurotransmitter secretion and the most upstream gene currently known in the gene cascade that underlies regulated secretion.

Ultrastructural evaluation of the munc18-1 homozygous mutants indicated that synapses are present in the mutant, that synaptic vesicles are targeted to these synapses, and that these vesicles dock at the site of release. In the prenatal neocortex at embryonic day 16 (E16) we found no effect of munc18-1 deletion in nerve terminal organisation (Fig.4). In the adult heterozygotes' neuromuscular junction, we also found no indications for morphological changes upon reducing munc18-1 expression (Fig.7). Despite this, there was

no secretion from these synapses. This absolute defect in secretion cannot be attributed to the loss of other proteins than munc18-1. Although we observed lower levels of 6 protein components of the secretion machinery in the absence of munc18-1, these were still abundant in the synaptic areas (Fig.4). Hence, their reduced levels cannot account for the complete loss of synaptic vesicle secretion. The docked state of synaptic vesicles is defined on a morphological basis. Estimates of docked vesicles may therefore include vesicles that lie adjacent to the membrane by chance. However, the distribution of vesicles, and especially of docked vesicles, in the mutant synapses can not be explained by assuming a random distribution. Hence, we conclude that munc18-1 mediates a biochemical step in regulated exocytosis that is downstream of vesicle docking.

Although there was no regulated exocytosis in the homozygous mutant, the data from the heterozygous animals indicate that vesicle fusion itself was not affected by lowering munc18-1 expression. We observed no changes in the release probability (Fig.6) or the Ca^{2+}-dependence of release (data not shown). We did observe a selective decrease in the number of synaptic vesicles that are available to undergo exocytosis in the heterozygous animals. This is reflected in four electrophysiological parameters (i) the frequency of spontaneous fusion events (ii) the number of quanta secreted per impulse and (iii) the response to hypertonic solutions to probe the releasable pool of vesicles, and (iv) the run-down of the synaptic responses upon frequent stimulation. Apparently, the munc18-1 expression level determines the amount of vesicles available for secretion (the fusion-ready vesicle pool). Hence, we conclude that munc18-1 functions to make synaptic vesicles fusion-ready, i.e., a biochemical step downstream of docking and upstream of fusion.

Recently, another synaptic protein, munc13-1 was implicated in a similar step ("synaptic vesicle maturation") in a subset of neurones (Augustin et al., 1999). Interestingly, munc18-1 and munc13-1 both bind to syntaxin-1 and also to DOC2. Although the two proteins are structurally unrelated, it was suggested that they might have similar functions, at least in some neurones. However, the fact that munc18-1 expression can not rescue the lethal phenotype of the munc13-1 homozygous mutant (Augustin et al., 1999) argues for different functions of the two proteins.

Genetic studies in *Drosophila* (Schulze et al., 1994; Wu et al., 1998) and biochemical studies *in vitro* (Pevsner et al., 1994a) suggested that one aspect of rop/munc18-1 function is a negative regulation of syntaxin-1 function. Our data clearly confirm that the presence of munc18-1 is important for normal syntaxin levels, suggesting that the interaction between the two proteins has physiological importance. Similarly, the reduction in cellular DOC2 levels suggests that also the previously observed interaction between DOC2 and munc18-1 (Verhage et al., 1997) has physiological importance. However, a modulatory (regulatory) role of munc18-1 cannot explain the complete loss of transmission in the homozygous mutants. Furthermore, reduced munc18-1 expression in the heterozygous mice leads to impaired secretion, especially during high activity. This argues against negative regulation. Together these data suggest that munc18-1 primarily acts as an essential (positive) effector in secretion and not as a (negative) regulator.

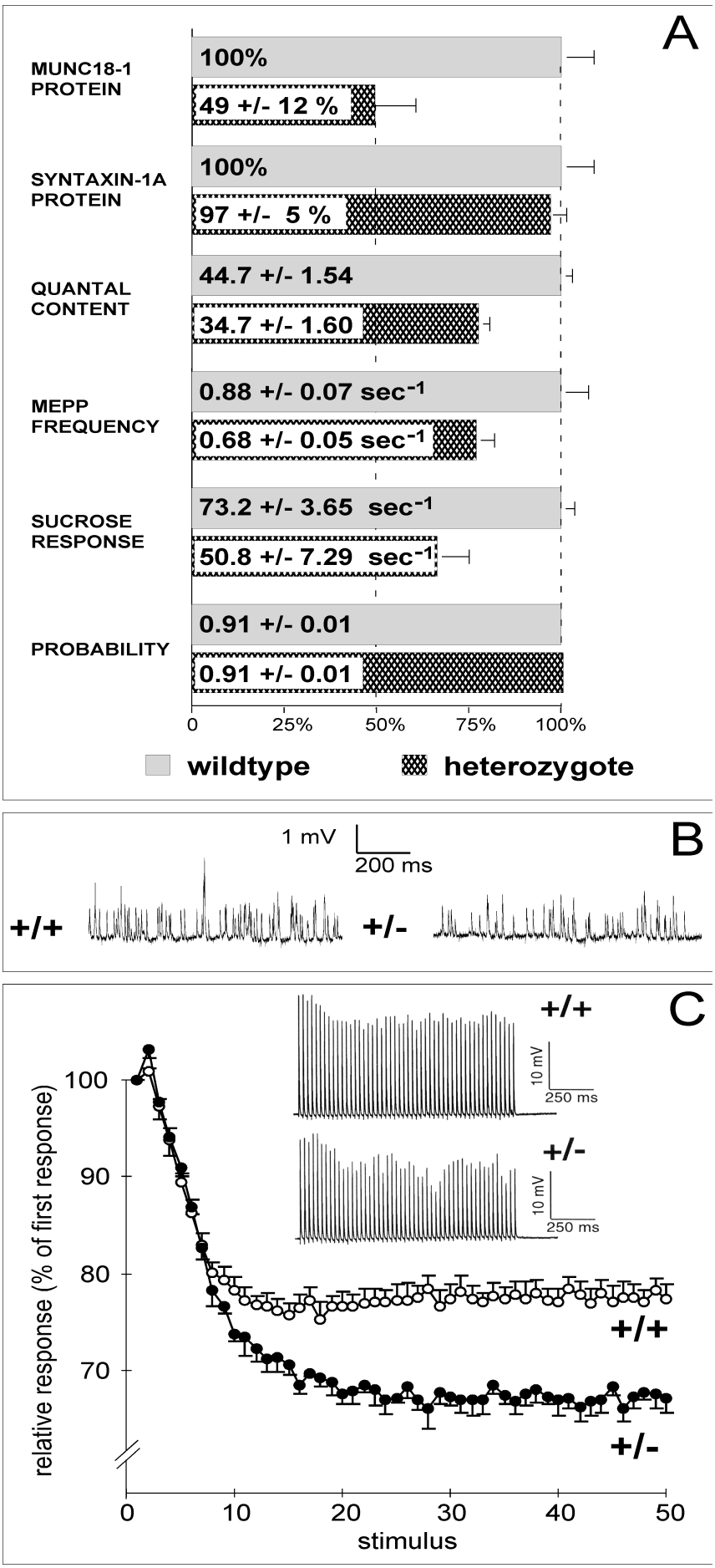

Fig.6) **A**) Several physiological parameters in heterozygous and wild type littermates compared to the munc18-1 protein levels. For each parameter, the wild type value was set at 100%. Where applicable, the absolute values are indicated in the bars. Note the almost proportional decrease in the sucrose response as compared to the decrease in munc18-1 protein level. Data represent means ± SEMs of 4-5 animals per group and 15-20 neuromuscular junctions sampled per animal. Differences between the groups were significant for quantal content ($p<0.002$), MEPP-frequency ($p<0.046$), sucrose response ($p<0.033$) and releasable pool n ($p<0.004$). **B**) Typical examples of sucrose responses in wild type and heterozygous littermates **C**) 40Hz Run-down of the evoked response in wild type and heterozygous littermates. Indicated are the responses to each individual stimulus for 50 stimuli delivered to the phrenic nerve at 40Hz, expressed as percentage of the initial response. Data represent means ± SEMs of 5 animals per group and 15 neuromuscular junctions sampled per animal. The inset shows a typical example of the run-down in each group.

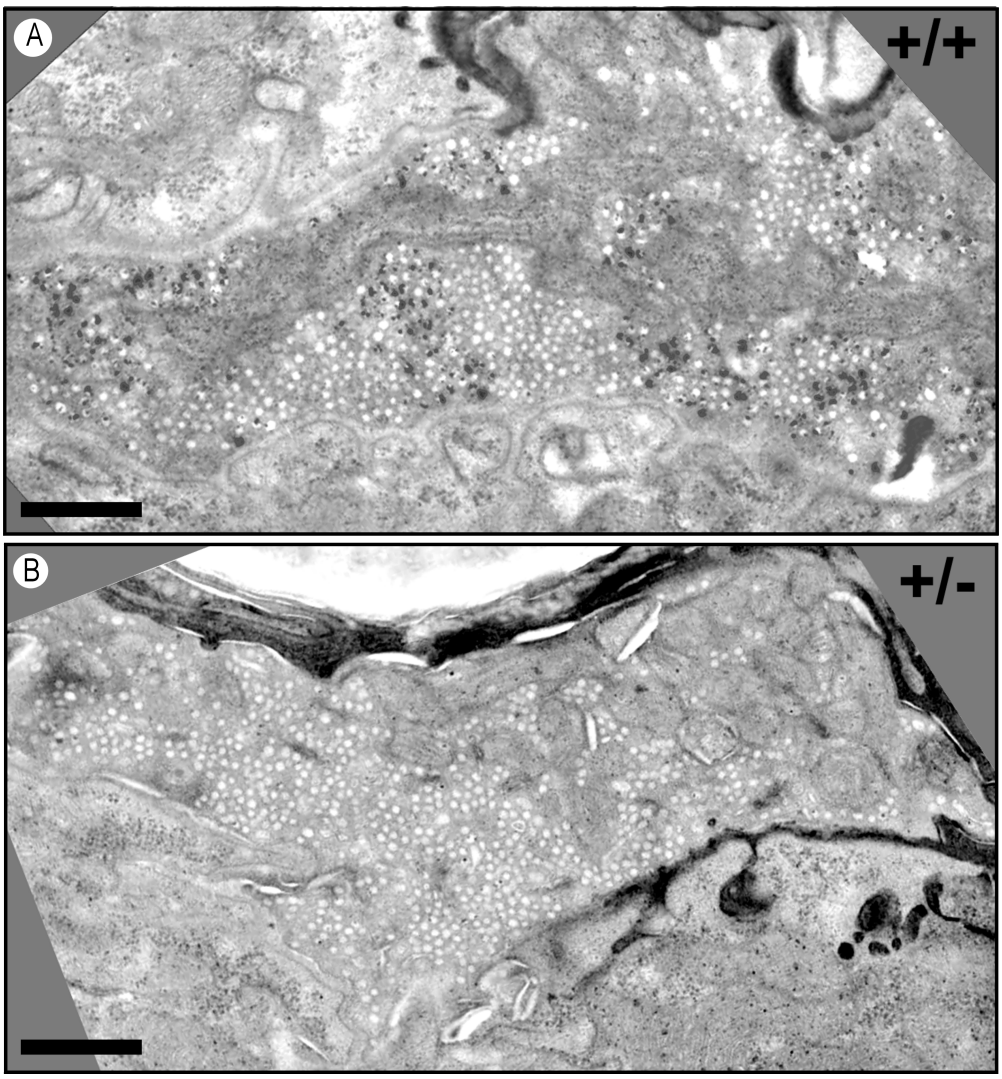

Fig.7) Ultrastructure of adult diaphragm neuromuscular junctions of wild types and heterozygotes. Typical examples of neuromuscular synapses of wild type (**A**) and heterozygous (**B**) animals showing abundant clustered and docked synaptic vesicles near/at synaptic complexes.
Bar: 100nm.

Notably, deletion of munc18-1 expression does not affect the cellular levels of synaptobrevin-II/VAMP2 and SNAP-25, two proteins that also bind syntaxin-1 and are believed to drive vesicle fusion as a trimeric complex.

Rabphilin-3A and synapsin were found at lower levels in the null mutant mice. In contrast to syntaxin-1 and DOC2, these proteins have not been found to physically interact with munc18-1. Rabphilin-3A and synapsin are peripheral proteins of the synaptic vesicle and dissociate from these vesicles as a function of exocytosis (Silva et al., 1996; Stahl et al., 1996). Rabphilin is also destabilised in rab3A mutants (Geppert et al., 1994; Li et al., 1994). The decreases in these proteins suggests that an active vesicle cycle or a stable protein complex may be required for their stability.

To analyze the synaptic function of munc18-1 it was essential that the neuronal degeneration that occur in the null mutant mice late in embryonal development (Verhage et al., 2000) do not hamper our interpretation. We have shown that in a brain area where no degeneration was found, the neocortex, synaptic transmission is also completely lost while synapses are readily formed. Furthermore, levels of synaptic vesicle proteins are normal and immunostaining for synaptic vesicle markers is found in synaptic areas of the brain. This indicates that generation of synaptic vesicles and targeting of synaptic vesicle proteins from the somata is not blocked by abolishing munc18-1 expression. Furthermore, indications for

the synaptic function of munc18-1 are largely based on data obtained from the heterozygous mice. The absence of degeneration and changes in the levels of proteins other than munc18-1 in these animals allows an unbiased characterisation of munc18-1 function. In these mice, we observed a selective and consistent decrease in several electrophysiological parameters that relate to the number of synaptic vesicles available for secretion, whereas other aspects of secretion were not changed.

In conclusion, genetic manipulation of munc18-1 expression in mice indicated that munc18-1 is an essential effector in regulated exocytosis. Without this gene, no aspect of regulated exocytosis, spontaneous or evoked, occurred. Its synaptic function is to mediate a biochemical step that determines the releasable pool of synaptic vesicles in the nerve terminal, downstream of docking and upstream of fusion. Munc18-1 is essential for the stability of several synaptic proteins, especially syntaxin-1.

chapter 3

Synaptic assembly of the brain in the absence of neurotransmitter secretion via synaptic vesicle

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ABSTRACT

Brain function requires the precisely orchestrated connectivity between neurones. Establishment of these connections is believed to require signals secreted from outgrowing axons, followed by synapse formation between selected neurones. Deletion of a single protein, munc18-1, in mice leads to a complete loss of neurotransmitter secretion from synaptic vesicles throughout development. However, this does not prevent normal brain assembly, including layered structures, fibre pathways, and morphologically defined synapses. After assembly is completed, neurones undergo apoptosis, leading to widespread neurodegeneration. Thus, synaptic connectivity does not depend on neurotransmitter secretion, but its maintenance does. Neurotransmitter secretion functions to validate already established synaptic connections.

Synapses are focal points of communication between nerve cells. Together, billions of synapses account for the unique connectivity of the brain (Sheperd, 1990). To establish the synaptic network, outgrowing axons are precisely directed to their targets using a variety of guidance cues and recognition signals on the outgrowing axon and the target cell (Keynes and Cook, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Fusion of neurotransmitter vesicles at the axon tip is believed to supply the membrane for axonal outgrowth (Catsicas et al., 1994; Osen-Sand et al., 1996; Williamson and Neale, 1998). The concomitant neurotransmitter release is thought to have a trophic role, and to provide essential signals for the correct targeting of axons and synapse formation: components of the presynaptic secretion machinery are already expressed in immature neurons before they differentiate (Fletcher et al., 1991; Daly and Ziff, 1997). These components are targeted to the axon tip where they are thought to be essential for axonal outgrowth *in vitro* (Matteoli et al., 1992; Osen-Sand et al., 1993, 1996; Catsicas et al., 1994; Igarashi et al., 1997; Williamson and Neale, 1998). Outgrowing neurons have an active synaptic vesicle cycle, secrete neurotransmitters prior to synapse formation, and upregulate this secretion once the growth cone comes close to its target (Xie and Poo, 1986; Sun and Poo, 1987; Coco et al., 1998). After synapses have formed, the secretion capacity of nerve terminals is upregulated, and the Ca^{2+}-affinity and tetanus toxin sensitivity increase.

Many genes that function in neurotransmitter secretion at mature synapses have been identified (Sudhof, 1995; Calakos and Scheller, 1996), and drastic phenotypes have been observed upon deleting such genes in mice (Fernandez-Chacon and Sudhof, 1999). However, all currently known gene-deletions show normal brain development. This can be explained by the fact that only certain aspects of the presynaptic function are abolished in these mutants. In particular, spontaneous, quantal transmitter release is retained even in the most severely affected mutants (Geppert et al., 1994b; Augustin et al., 1999). We have now identified the munc18-1 gene as essential for all components of neurotransmitter release throughout the brain. Munc18-1 is a neuron-specific protein of the SEC1-family of membrane trafficking proteins (Hata et al., 1993; Garcia et al., 1994; Hata and Sudhof, 1995; Tellam et al., 1995) and is expressed throughout the brain and interacts with at least three classes of proteins, suggesting it may regulate cell polarization as well as focal secretion at synapses (Okamoto and Sudhof, 1997; Verhage et al., 1997; Butz et al., 1998). We have abolished munc18-1 expression in mice by homologous recombination (Fig.1). This resulted in a completely paralysed organism. Null-mutant embryos are alive until birth, but die immediately after, probably because they cannot breathe.

Synaptic transmission can normally be detected as soon as a synapse is formed. In the mammalian neocortex, the first synapses are observed at E16 (Konig et al., 1975). Electron microscopy of the marginal zone in control embryos confirmed the presence of a few synapses at E16, although we did not detect synaptic secretion events in neocortical slices at E17. However, at E18, synaptic events were readily observed (Fig.2A). In contrast, null-mutants lacked synaptic events (Fig.2A). Nevertheless in these mutants, postsynaptic receptors were functional (Fig.2B), spontaneous action potentials were occasionally observed (Fig.2C), and ion-channels showed normal properties (Fig.2D). To study neurotransmitter secretion in a different, earlier developing synapse, we investigated the diaphragm neuromuscular junction between E15 and E18 (16). In these synapses too, synaptic events were detected in controls, but null-mutants lacked synaptic events at E15, E16, and E18 (Fig.2E). Application of α- latrotoxin or electrical

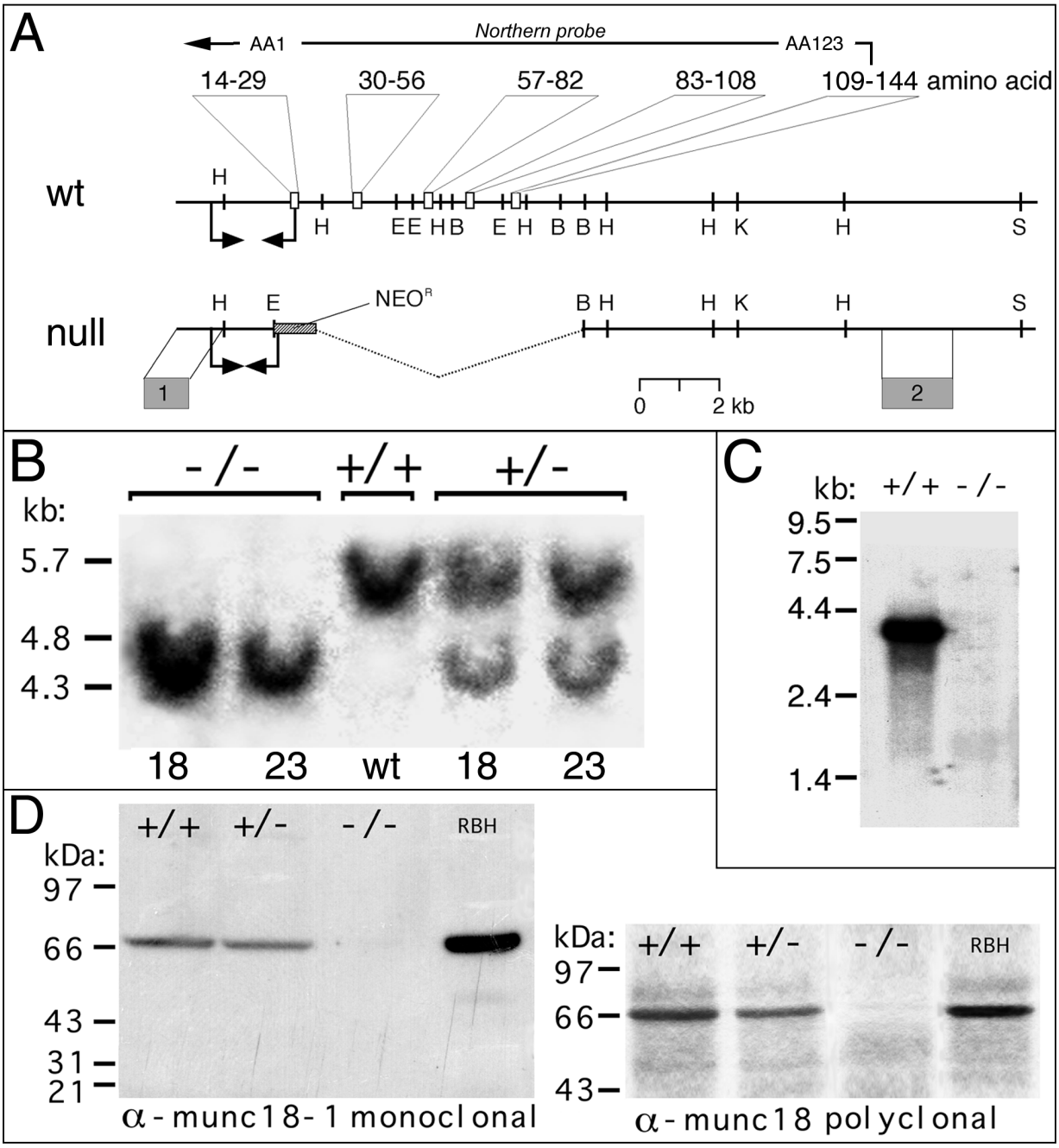

Fig.1) Generation of mice lacking munc18-1. **A**) Gene structure of wild type munc18-1 (wt) and after homologous recombination (null). Boxes indicate exons with encoding amino acid numbers above. Five exons are replaced with a neomycin resistance gene (NEOR). Arrow-pairs indicate PCR primers for genotyping. The top arrow indicates the Northern probe and the grey boxes indicate Southern probes. H, HindIII; E, EcoRI, B, BamHI; K, KpnI; and S, SmaI. **B**) Southern blot of munc18-1 mutants. EcoRI-digested genomic DNA from wild types (+/+), heterozygotes (+/-), and homozygotes (-/-) was hybridised with probe 1. Two independent mouse lines (called 18 and 23 after the ES cell clone) contained identical deletions. **C**) RNA blot of munc18-1 mRNA in wild types and homozygotes around birth. **D**) Immunoblot analysis of munc18-1 at E18 in all genotypes. Blots were probed with a munc18-1-specific monoclonal antibody (left) or a general munc18 polyclonal antibody (right) to detect possible compensatory changes in other munc18 isoforms. RBH: rat brain homogenate.

nerve stimulation strongly stimulated synaptic transmission in control littermates but caused no response in null-mutants, although their postsynaptic receptors were functional (Figs.2E & F).

Despite the general, complete and permanent loss of synaptic transmission in the knockout mice, their brains were assembled correctly (Fig.3). Neuronal proliferation, migration and differentiation into specific

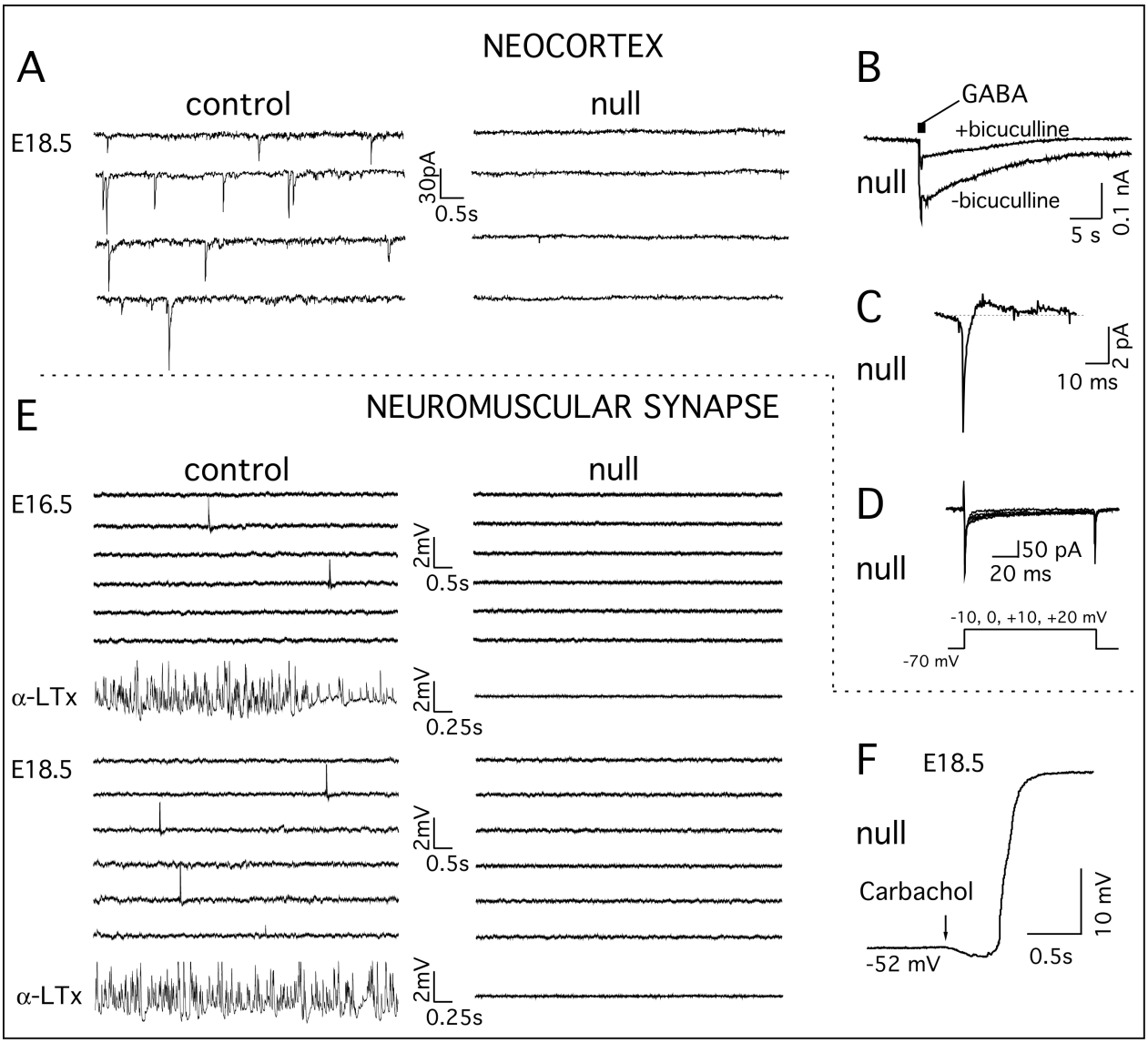

Fig.2) Munc18-1 deficient mice lack synaptic neurotransmitter release. **A**) Whole-cell voltage-clamp recordings from neocortical neurons in slices at E18. Wildtype neurons showed frequent spontaneous synaptic events (5.4 ± 2.4 min^{-1}; mean ± SEM, n=3 animals, 4 recordings) while neurons in null-mutant slices were completely devoid of such activity (n=3 animals, 5 recordings, total recording: 30.8min). **B**). GABA-iontophoresis (1M, 200-400nA, 10-20 pulses for 0.1s at 4Hz) in mutant brain slices caused a normal postsynaptic response, sensitive to bicuculline (20μM in bath). **C**) Spontaneous action potentials in the cell-attached mode. **D**) Voltage-gated inward ion currents in whole-cell mode in mutant cells. (C) and (D) indicate that we recorded from neurons, not glial cells. **E**) Intracellular recordings in diaphragm musclefibres from control and null-mutant littermates. Control embryos exhibited miniature endplate potentials (MEPPs), 5.4 ± 0.6min^{-1} at E15 (1 animal, 9 fibers ± SEM); 1.3 ± 0.3min^{-1} at E16 (2 animals, 5 fibers per animal ± SEM), and 2.8 ± 0.6 min^{-1} at E18 (2 animals, 5 fibers per animal ± SEM). No MEPPs were detected in munc18-1 deficient mice at E15 (9 fibers, total recording: 18.8min), E16 (5 fibers, 21.5min recording) and E18 (11 fibers, 35.5min recording). α-Latrotoxin (4nM) induced massive neurotransmitter release in controls, but was completely ineffective in mutant muscle fibers. F. Carbachol (1mM) elicited a strong postsynaptic potential and contraction in E18 mutant muscle fibers.

brain areas were unaffected. At E12, brains from null-mutant and control littermates were morphologically indistinguishable (Figs.3A & B). At birth, late-forming brain areas such as the neocortex appeared identical in null-mutant and control littermates, including a distinctive segregation of neurones into cortical layers (Figs.3C & D). Furthermore, fibre pathways were targeted correctly in null-mutants: The growth cone marker GAP-43 showed a normal distribution, including marked staining of fibre bundles and synaptic layers (Fig.3G & H). Immunolabelling patterns for presynaptic markers were similar in mutant and control littermates (Figs.3I & J). The levels of several synaptic proteins were also normal in the mutants, suggesting

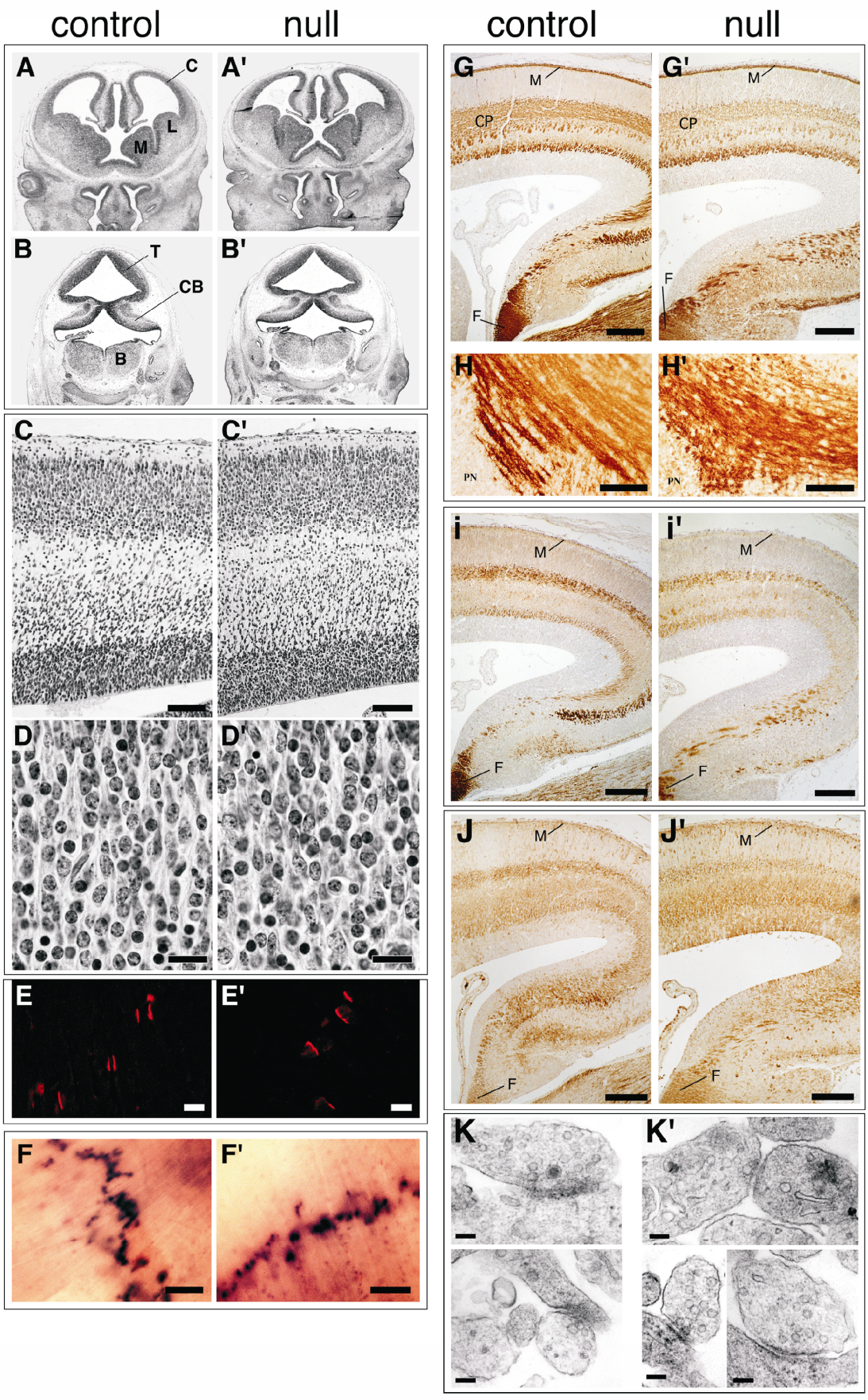

Fig.3) Correct assembly of the brain in the absence of neurotransmitter secretion. **A** & **B**) Coronal sections of developing brains

at E12 from control (A, B) and null-mutant (A', B') littermates stained with hematoxylin/eosin. (A) is located anterior to (B). C: cortex, CB: cerebellar anlage; T: tectum; L and M: lateral and medial ganglionic eminence; B: brainstem. **C** & **D**) Normal architecture of the neocortex in null-mutant mice at E18. Bars: 100μm (C), 20μm (D). **E**) Clustering of acetylcholine receptors in the diaphragm neuromuscular junction visualised with fluorescent α-bungarotoxin at E18. Bar: 2.5μm. **F**) Accumulation of acetylcholine esterase at synaptic sites in the diaphragm at E18. **G** & **H**) GAP-43 staining of the developing cortex at E18 (G) and the giant fiber pathway above the pontine nucleus (PN). M: marginal zone (synaptic layer); F: fimbria fornix fiber bundle. Bar: 200μm (G), 20μm (H). **I** & **J**) Synapsin I (I) and synaptobrevin/VAMP II (J) staining at E18, showing that synaptic vesicle markers are transported to the synaptic layer (M). Bar: 200μm. **K**) Synapses in the neocortex marginal zone at E16 showing apparently normal synaptic structures in the null-mutants (K') with clustered and docked synaptic vesicles near/at presynaptic active zones and postsynaptic densities. Bar: 50nm.

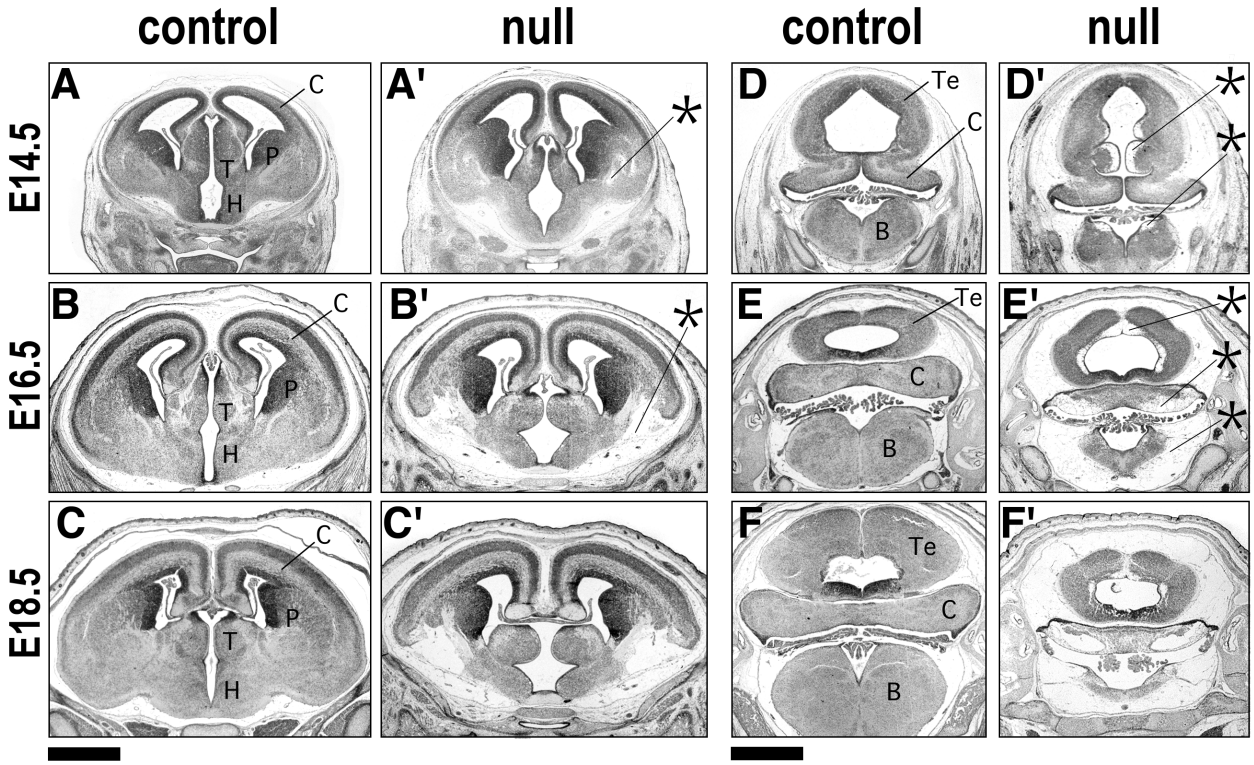

Fig.4) Massive neurodegeneration after assembly of the neuronal networks. Coronal brain sections of control (**A-F**) and null-mutant (**A'-F'**) littermates between E14 and birth. The asterisks identify locations where degeneration starts. Bar: 1mm. C: cortex; CB: cerebellar anlage; B: brainstem; H: hypothalamus; P: putamen; T: thalamus; Te: tectum.

that neuronal differentiation and synthesis of synaptic components proceeded normally. Moreover, synapses were readily formed in null-mutants, in the neocortex already at the onset of synaptogenesis at E16 (Konig et al., 1975). These synapses exhibited all signs of synaptic complexes (Fig.3I). The synapses in controls and knockouts contained the same numbers of total and docked synaptic vesicles (see chapter 4). Other ultrastructural aspects were also indistinguishable between mutants and controls (Sanes and Lichtman, 1999).

Formation of neuromuscular synapses requires precise navigation of nerve terminals over large distances. At the neuromuscular junction, nerve terminals secrete signals such as agrin that induce clustering of postsynaptic acetylcholine receptors and acetylcholinesterase (DeLaPorte et al., 1998). Thus, assembly of receptor- and enzyme clusters provides a sensitive measure of pathfinding and synaptogenesis. α-Bungarotoxin and acetylcholinesterase staining of diaphragm muscles revealed clear receptor- and enzyme-clusters in null-mutant mice (Fig.3E & F), suggesting that long-range axonal pathfinding and initial synapse formation must have occurred.

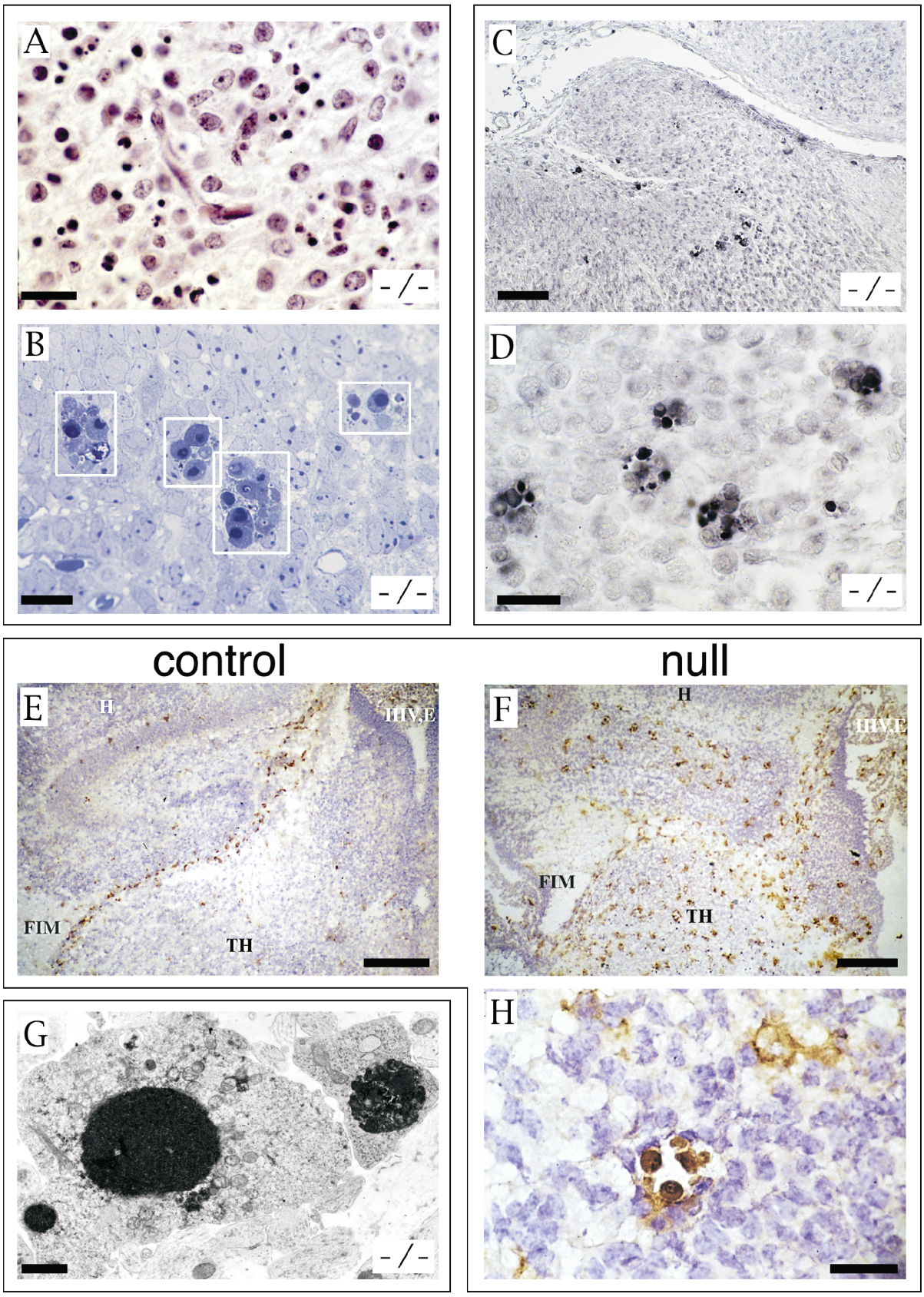

Fig.5) Neurones undergo massive apoptosis after initial synaptogenesis. **A** & **B**) Cell death exhibiting dark, indented nuclei in the thalamus of null-mutant mice at E18 stained with hematoxylin-eosin (A, see methods histology and immunohistochemistry) or methylene blue (B, see methods electron microscopy). Bar: 20µm. **C** & **D**) TUNEL staining of null-mutant hippocampus. Bar: 200µm (C), 20µm (D). **E**, **F** & **H**) Activated macrophage staining in the hippocampus/thalamus at E18 using F4/80 antibody. In

controls, activated macrophages were largely restricted to the ventricles (E). In the null-mutants, dense staining was observed throughout the tissue and colocalised with apoptotic cell bodies (panel F & H) that were probably engulfed by macrophages (panel H). H: hippocampus, FIM: fimbria, Th: thalamus. Bar: 200μm (E,F), 20μm (H). **G**) Electron micrograph of an affected neurone in the null-mutant thalamus showing compacted chromatin inside the nucleus. Bar: 3μm.

After initial brain assembly, extensive cell death of mature neurones was observed in the null-mutants (Fig.4), occurring first in lower brain areas that mature and form synapses relatively early (Fig.4D & F). For example, at E12 the brainstems of null-mutant and control littermates were morphologically similar (Fig.3B). While this area expanded further in controls, the neurones disappeared in null-mutants until, at E18, the brainstem was almost completely lost. Degeneration occurred later in the midbrain and basal forebrain (Fig.4A & C). Brain areas that develop last, especially the neocortex, were indistinguishable from control at birth. The degeneration in the mutant brains exhibited all characteristics of apoptosis: apoptotic bodies were readily observed with standard histology (Fig.5A & B). These were positive in TUNEL (TdT-mediated dUTP nick end labelling) staining (Fig.5C & D). Apoptotic bodies contained condensed chromatin (Fig.5G). A final phase of these degeneration was accompanied by abundant staining for activated macrophages (Fig.5E, F & H), and disintegration of lower brain areas around birth (Fig.4).

Thus, a gene-deletion that abolishes presynaptic secretion and herewith synaptic transmission, allows an apparently normal assembly of the brain. This is quite unlike gene-deletions for developmental factors, which generally result in some defect in the assembly of the brain (agenesis) (Joyner, 1996; Tanabe and Jessell, 1996; Hallonet et al., 1998). Ablation of munc18-1 renders the brain synaptically silent, identifying munc18-1 as the currently most upstream essential protein in neurotransmitter release. Axons in the munc18-1 mutants extend normally and form precise connections between widely separated brain areas, and synapses develop that look morphologically normal. It has been proposed that early spontaneous activity of neurons results in Ca^{2+} transients and may be critical for proper differentiation and pathfinding (Gomez and Spitzer, 1999; Milner and Landmesser, 1999). However, this spontaneous activity must act bya mechanism that does not require synaptic neurotransmitter release because munc18-deficient neurones differentiate normally, exhibit spontaneous action potentials, and perform apparently normal pathfinding. After synaptic assembly of the brain, activity-dependent selection is thought to maintain certain synaptic connections for adult life while others are discarded (Sheperd, 1990; Keynes and Cook, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Our data indicate that the neuronal networks are synaptically assembled and reach the selection stage without synaptic transmission, but cannot persist without it. When synaptic transmission is absent in newly established synaptic connections, these synapses degenerate, and the neurons go into apoptosis. Finally, our data indicate that the distribution of membrane to the growing axon tip and the release of signals that allow correct axon targeting depend on different molecular mechanisms than neurotransmitter release by regulated exocytosis of synaptic vesicles.

METHODS

Homologous recombination

Two murine genomic munc18-1 clones in λ-FIX were used to construct a targeting vector to replace five exons of the munc18-1 gene by a neomycin-resistance gene flanked by an 11.5Kb long arm and a 1.4Kb short arm, which in turn is flanked by two copies of the Herpes simplex thymidine kinase gene. ES cells ("G-cells", gift of J. Herz, Dallas) were electroporated (Rosahl et al., 1993) and analysed using PCR with oligonucleotide A: outside sense primer CGGTACTTGGGGATTGAACCCAGGC; B: Neomycin antisense

primer to detect the mutant allele GGATGCGGTGGGCTCTATGGCTTCTGA; and C: inside antisense primer to detect wildtype allele, AAAGGAACGGGGTGGAGGGAGAGA. Homologously recombined clones were confirmed by Southern blotting with outside probes (A and B in Fig.1A). Two positive ES cell clones were injected into blastocysts, generating highly chimeric mice that transmitted the mutation through the germline. The genotypes of litters from heterozygote matings exhibited a Mendelian distribution.

Electron microscopy

Brains of E16-18 embryos were immersed in 1% paraformaldehyde, 2% glutaraldehyde, 0.05M sodium cacodylate buffer pH7.4 for 2 days at 4°C, post-fixed in 1% OsO_4 in 0.1M cacodylate buffer, dehydrated, and embedded in EPON. Ultrathin (90nm) sections were contrasted with uranyl acetate/lead citrate. Synapses were defined as structures containing 1 or more 30-50nm vesicles in the vicinity of a pre- and postsynaptic specialisation. For panel B of Fig.5, brains were sectioned at 5μm and post-stained with methylene blue.

Electrophysiology

Cortical slices (400μm) at E17 and E18 were prepared on a Camden vibratome and whole cell recordings were performed *in situ* at 33°C and a holding potential of –70mV (Brussaard et al., 1997). Neuromuscular junction recordings were performed on diaphragm nerve/muscle preparations at E15, E16, and E18 using 30-40MΩ glass capillary microelectrodes at 26-28°C (Plomp et al., 1994). The phrenic nerve was stimulated via a suction electrode; responses were recorded intracellularly in muscle fibres at endplates. Thereafter, 1μM tetrodotoxin was added to suppress spontaneous contractions of fibres which occurred in all genotypes and interfered with recording of spontaneous events. In control experiments, a micropipette with 1mM carbachol and a broken tip to allow leakage was brought into the vicinity of the measuring electrode.

Quantification of electron micrographs from the neocortex marginal zone at E16: Synaptic vesicles per synapse, wild type: 5.2 ± 2.5; null-mutant: 3.9 ± 1.7. Docked synaptic vesicles per synapse, wild type: 2.1 ± 1.4; null-mutant: 1.9 ± 1.1 (3 animals, 3 sections per animal ± SEM; one section = ±1700μm^2).

Histology and immunohistochemistry

Throughout this study, mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous matings. Littermates were analysed without prior genotyping. Null-mutant animals had a beating heart until birth. Data from wild type and heterozygous embryos were pooled as the control group after pilot experiments revealed no significant differences between these groups. Animals were housed and bred according to institutional, Dutch and US governmental guidelines.

Mouse embryos (E12-18) were immersed in 71.4% saturated picric acid, 23,8% formalin, 4.8% acetic acid for 3 days, dehydrated, and embedded in paraffin. Brain sections (5μm) were stained with hematoxylin/eosin. For immunohistochemistry, sections were rehydrated, microwaved in 0.1% Froggy® detergent in 50mM TBS pH7.5 (4x 5min) and washed 3x in TBS. Endogenous peroxidase was blocked with 0.6% H_2O_2 in 100% methanol for 30min at room temperature. Sections were washed again 3x in TBS, and incubated for 1h at room temperature in 3% normal goat serum (NGS), 250mM TBS, 1% BSA, 0.1% Triton-

X-100. For monoclonal antibodies, NGS was replaced by 1% goat anti-mouse serum. Section were incubated at room temperature with primary antibodies overnight, with biotinylated secondary antibody for 1h, with peroxidase-labelled streptavidin-biotin complex for 1h, and with 3',3'-diaminobenzidine. For the monoclonal synaptobrevin antibody the Sternberger PAP-method® was used for detection. Antibodies used: 9527 (GAP-43), CL69.1 (synaptobrevin II), and E028 (Synapsins). Control experiments omitting primary antibodies confirmed staining specificity.

Acetylcholine receptors and acetylcholine esterase were analysed in whole mount diaphragm neuromuscular junctions at E18. Diaphragms were fixed (90min, 2% paraformaldehyde in PBS) and rinsed (30min in 0.1M glycine in PBS). After 15min pre-incubation in 0.5% Triton-X-100 and 1% BSA in PBS, 1 μg/ml TRITC-labeled α-bungarotoxin was added, and diaphragms were incubated overnight at 4°C. Diaphragms were washed, mounted in Dabco-Mowiol and analysed with confocal laser microscopy. Acetylcholine esterase was stained in unfixed diaphragms 1h with 0.5mM 5-bromo indoxylacetate.

Cryosections of embryonic mouse heads (10μm) were fixed for 10min in acetone, incubated with monoclonal antibody F4/80 (undiluted cell supernatant, Hume and Gordon, 1983) for 1h at room temperature, rinsed 3x with PBS, incubated with peroxidase-coupled rabbit anti-rat antibody for 1h, rinsed again 3x with PBS and stained with 3',3'-diaminobenzidine. Sections were washed in 0.9% NaCl, incubated in $CuSO_4$ for 30min, washed again, immersed in hematoxylin, and dehydrated.

Protein analysis

Protein quantification in total brain homogenates from E18 embryos was performed by quantitative immunoblotting using ^{125}I-labelled secondary antibodies (McMahon et al., 1996). To correct for degeneration in the null-mutants (Fig.4 & 5), protein levels were corrected using hexokinase, GDI, and calmodulin as internal standards. Relative protein levels in knockout animals compared to wild type (100%) were (mean ± SEM from triplicate determinations): synaptophysin, 96 ± 14%; synaptobrevin II, 98 ± 12%; rab3A/C, 87 ± 16%; GAP-43, 95 ± 10%; and NMDA-receptor, 101 ± 5%.

TUNEL staining

For TUNEL staining, paraffin sections (see histology and immunohistochemistry) were rehydrated, incubated with 1% H_2O_2 in methanol for 5min, microwaved in 1% $ZnSO_4$, 1% Triton in 10mM PBS at 45°C/150W for 5min, rinsed at 4°C, and microwaved and rinsed again. Sections were stained with *In situ* Cell Death Detection Kit, peroxidase-conjugated converter enzyme POD and 3',3'-diaminobenzidine-Ni^{3+}. As controls either TUNEL enzyme or POD converter enzyme were omitted.

chapter 4

Quantification and characterization of synapse formation and maintenance *in vivo* in the absence of synaptic vesicle secretion

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ABSTRACT

Outgrowing axons in the developing nervous system secrete neurotransmitter quanta. This is considered to be a trophic factor for synaptogenesis, but some synapses also appear to form independent of neurotransmitter secretion. We analyzed synapse formation and maintenance quantitatively in the neocortical marginal zone of a mutant mouse lacking both evoked and spontaneous quantal secretion (Verhage et al., 2000). The axon-dendritic network and the number of cell bodies and large dense core vesicles were similar to control littermates. At embryonic day 16 (E16), synapses had similar size and active zone length and contained similar amounts of total and docked synaptic vesicles. However, synapses were 3 fold less abundant in the mutant. Two days later, synapses in the mutant contained fewer vesicles and the difference in abundance increased to 5 fold. Conversely, structures characterized by double electron-dense membranes with irregular shaped vesicles on both sides were 4 fold more abundant in the mutant. These structures were also labeled for synaptobrevin/VAMP and rab3. These data are compatible with the concept that without secretion, synapse formation is unimpaired but that synaptic activity is required for subsequent maturation/stabilization. Without sufficient activity, synapses disassemble, resulting in multivesicular structures. We suggest that spontaneous quantal secretion is sufficient to prevent disassembly.

INTRODUCTION

In the developing nervous system, outgrowing axons secrete neurotransmitters, such as acetylcholine and GABA, from their growth cones in a quantal manner (Xie and Poo, 1986; Sun and Poo, 1987; Gao and Van Den Pol, 2000). This process is considered to have trophic influences on synapse formation and secretion machinery manipulation affects axon outgrowth and synapse formation (Han et al., 1991; Osen-Sand et al., 1993; Kabayama et al., 1999). However, a variety of studies wiht cultured neurons suggest that at least propagation of action potentials and the activation of neurotransmitter receptors are not necessary for the formation of normal synaptic networks *in vitro* (see for instance Van Huizen et al., 1985; Verderio et al., 1994, but note Nakagami et al., 1997). Furthermore, synaptic activity also appears not to be essential for synapse formation in certain cases *in vivo*. Retinotectal projections form normally after eliminating impulse activity in amphibians (Harris, 1980), neuromuscular junctions are correctly formed in the absence of postsynaptic neurotransmitter receptors in fish (Westerfield et al., 1990) and the synaptic connectivity of the nervous system of *C. elegans* forms normally in the absence of one of its most abundant neurotransmitters (Jin et al., 1999). Finally, we have recently shown that the mouse brain is assembled correctly, including the formation of synapses, in the absence of quantal secretion (chapter 3).

Still, synaptic activity has a certain role in establishing mature neuronal networks. When transmission is blocked, *in vitro* networks mature more slowly (Van Huizen et al., 1985) and massive cell death occurs *in vivo* (Fawcett et al., 1984; Ikonomidou et al., 1999; chapter 3). Furthermore, synaptic activity is known to be important in reshaping the multiple innervation in the neuromuscular junction (see Lichtman and Colman, 2000) and more complex reshaping of existing networks, for instance in the visual system (Chapman et al., 1986; Hayes and Meyer, 1989; Goodman and Shatz, 1993). Hence, initial synapse formation may not absolutely require transmission, at least in some systems, but subsequent phases of synapse maturation probably do. Thus, it is still unclear exactly when and how synaptic transmission influences the synaptic connectivity of the brain.

In addition to stimulus-evoked secretion of neurotransmitters, nerve terminals continuously secrete transmitters by spontaneous fusion of vesicles with a low incidence. The function of this spontaneous vesicular secretion for the development of the brain remains elusive, especially since no pharmacological tools are available to block spontaneous secretion.

To characterize the role of synaptic transmission and spontaneous vesicular secretion during synaptic development of the mammalian brain, we performed a quantitative morphometric analysis of synapse formation *in vivo*, in the neocortex of the mouse. We have exploited the fact that the presynaptic protein munc18-1 is essential for all aspects of vesicular secretion, evoked and spontaneous, throughout the brain and throughout development (chapter 3). We have previously shown that in munc18-1 deficient mice, the brain assembles correctly, including the formation of all the major pathways and nuclei in the brain and also observed developing synapses (chapter 3). In the present study, we have quantified and characterized synapse formation and maintenance in the neocortical marginal zone at two different time points during prenatal development. Control and mutant animals had initially similar synapse morphology, but controls had more synapses than mutants. Close to birth, synapse morphology differed, controls had more synaptic vesicles per synapse and higher numbers of synapses per unit area, but both parameters remained unchanged in the mutants. Instead, mutants had more of another, multivesicular structure, which may reflect disassembling synapses. Given the fact that other mutant mice showed no large scale disassembly

when evoked, but non-spontaneous vesicular secretion was blocked, we suggest that spontaneous, infrequent fusion of synaptic vesicles may already be sufficient to prevent disassembly at this developmental stage.

METHODS

Animals

Generation of germline-transmitting munc 18-1-deficient mice and PCR genotyping are described in chapter 2 and 3. Briefly, exons 2 to 5 of the munc 18-1 gene were replaced with a neomycin resistance gene using homologous recombination. The mouse colony was maintained by breeding of heterozygous animals. Heterozygous males were mated with synchronized heterozygous females for one night. If a vaginal plug was detected the following morning, this was considered embryonic day zero. Embryos were harvested from mothers rapidly sacrificed after timed matings at E16 and E18. Embryo heads were used for electron microscopy and a posterior paw was sampled for genotype analysis. Experimental procedures were carried out with local regulatory approval for animal experimentation.

Electron microscopy preparation

E16 and E18 mouse embryos were decapitated. In order to give fast access to the brain for the fixative a few drops of Karnovsky fixative (Karnovsky, 1965) (4% paraformaldehyde, 5% glutaraldehyde, 5mM $CaCl_2$, 10mM $MgCl_2$, 0.1M Na-Cacodylate pH7.4) or tri-aldehyde fixative (2,5% DMSO, 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolin in 0.1M Na-Cacodylate pH7.4) were poured onto the heads and the skin and skull were peeled off. The brains were severed sagittally between the two hemispheres. The heads were then put in tri-aldehyde fixative for 1h at room temperature and overnight at 4°C. The next day, further dissection was carried out, as indicated in Fig.1. Afterwards, a protocol described elsewhere was followed (Gorgels, 1991). Briefly, the area of interest was post fixed for 2h at room temperature in OsO_4 2%-$KRuCN_6$ 4% in cacodylate buffer 0.4M. After dehydration, the area of interest was embedded in EPON. For a consistent sampling of the same brain area, thin sections were made and controlled after toluidine blue staining. Ultrathin sections were then mounted on non-coated grids and contrasted with uranyl acetate/lead citrate. Four animals per group at each sample point were used and at least three non-consecutive sections per animal were examined, representing more than 5000μm^2.

Immunogold labeling

E18 mouse embryos were decapitated. We followed the same procedure as described above except that the fixative was 2% paraformaldehyde/0.2% glutaraldehyde in 0.1M PB, following a protocol described elsewhere (Geuze et al., 1981; Slot et al., 1988, 1991). Briefly, the embryos remained in the fixative for 2h at room temperature and overnight at 4°C. The area of interest was further dissected into small blocks and washed in PBS with 0.02M glycine. Blocks were embedded in 12% gelatin in PBS and solidified on ice. After infiltration with 2.3M sucrose at 4°C, blocks were mounted on aluminum pins and frozen in liquid nitrogen. Ultrathin sections were picked up in a 1:1 mixture of 2% methyl cellulose and 2.3M sucrose which improved ultrastructural preservation (Liou et al., 1996).

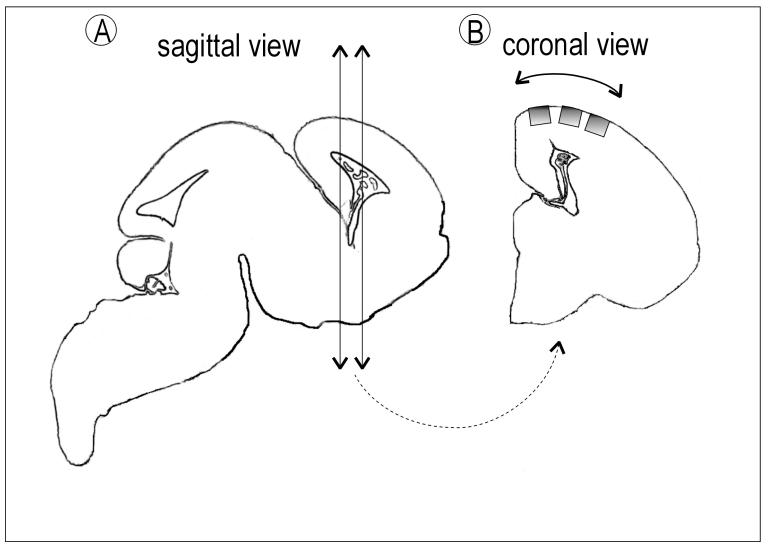

Fig.1) Scheme showing the dissection procedure used for a consistent sampling of the neocortical marginal zone (layer I) of the mouse embryos. **A**) Sagittal view of the brain. Double sense arrows indicate the levels of sectioning resulting in the thick slice visible in B. **B**) Coronal view of the brain slice obtained after sectioning. Double sense arrow indicates the area where quantification in EPON section occurred. Dark squares indicate the small blocks taken for immunogold labelling (see methods section).

Mouse monoclonal antibodies against VAMP/synaptobrevin (Cl69.1; (Edelmann et al., 1995) and Rab3 (Cl42.1 (Matteoli et al., 1991) were obtained from Synaptic Systems (Göttingen, Germany). Immunogold labeling was performed using gold particles-protein A conjugated to rabbit-anti-mouse IgG (DAKO, Denmark), as described previously (Slot et al., 1991).

Data collection and statistical analysis

The synaptic parameters chosen (see text) were quantified directly from the microscope's calibrated scale. The total areas scanned during these quantification were also measured directly from the electron microscope. Low magnification photos (x1200) of the cortical marginal zone were taken and used for the extracellular space and cell number measurements. A cell somata was counted only when a cell nucleus was visible. Extracellular space measurement was done following the stereology method described by Steer (Steer, 1981). Briefly, intersection points of crossing lines falling in the extracellular space were expressed as a percentage of the total intersection points comprised in the measured area. The raw data were analyzed on log-transformed data using Student's t-test with 2-sided p value. Statistical significance was accepted at $P<0.05$. Values are given as mean$\pm$ SEM.

RESULTS

Mutant synaptic layer, the cortical marginal zone, is normal

To investigate synaptogenesis, we chose the marginal zone of the neocortex. Some brain areas in the munc18-1 mutant mouse showed signs of degeneration, especially in the rhombencephalon, but the entire cortex of the mutant and control were alike until birth, with no conspicuous cell degeneration and showed similar staining for B50/GAP43, syntaxin I and VAMP/synaptobrevin (chapter 3). Furthermore, cortical plate cells, which project climbing axons to the marginal zone, and Cajal-Retzius cells do not need external input to differentiate (Bolz et al., 1996; Marin-Padilla, 1998; Miyashita-Lin et al., 1999). Thus, marginal zone synapses are formed by cortical neurons and not by neurons from other brain areas and the quantification of synapse formation will not be disturbed by the fact that some lower brain areas degenerate in the

mutant.

Using a low magnification at the electron microscope level, we analyzed control and mutant marginal zone ultrastructure and observed no differences. The marginal zone was composed of few somata and many axons and dendrites (Fig.2A & B). At E18, the marginal zone contained a similar number of cells in both groups, Cajal-Retzius cells and migrating or uncommitted neurons, which could not easily be discriminated, (Konig et al., 1981; Marin-Padilla, 1998); control 3.51 ± 0.60 per 1000μm^2; mutant 3.79 ± 0.34; mean ± SEM, n=4; Fig.2C). The percentage of extracellular space in the marginal zone was also similar in both groups (control 19.6 ± 5.3%; mutant 20.2 ± 3.5%; mean ± SEM, n=4; Fig.2D). Thus, the overall organization of mutant and control marginal zones appeared to be similar.

We also quantified the number of large dense cored vesicles (LDCV) in the marginal zone. Both groups had similar amounts of LDCV at E16 (control 34.2 ± 8.1 per 1000μm^2; mutant 30.8 ± 5.2; p=0.805) and at E18 (control 54.9 ± 12.8 per 1000μm^2; mutant 37.5 ± 4.9; Fig.3U).

It was previously shown that in the absence of munc18-1 protein and upon overexpressing syntaxin-1A protein, Golgi and rough endoplasmic reticulum underwent deformation. Expressing munc18-1 protein prevented such deformations (Rowe et al., 1999). This suggests that removing munc18-1 may produce intrinsic cellular abnormalities. However, analysis of the cortical plate cells of the munc18-1 mutant did not reveal such abnormalities.

At E16, synapse morphology was similar in controls and mutants

Examining the marginal zone at high magnification revealed that controls and mutants contained developing synapses (Fig.3A-O, see also chapter 3). We characterized synapse morphology by quantifying the presynaptic diameter, the active zone length and the total and docked vesicles. Control and mutant had comparable presynaptic diameter (control 0.68 ± 0.04μm; mutant 0.73 ± 0.09μm), active zone length (control 0.26 ± 0.01μm, mutant 0.27 ± 0.02μm), total amount of synaptic vesicles (control 4.5 ± 0.9; mutant 5.39 ± 1.6) and docked synaptic vesicles (control 1.2 ± 0.5; mutant 1.21 ± 0.4) per synapse (Fig.3P-R). Thus, synapse morphology at E16 of controls and mutants were similar.

At E18, synapse morphology differed between controls and mutants

At E18, presynaptic element diameter (control 0.58 ± 0.04 μm, mutant 0.65 ± 0.02 μm) and active zone length (control 0.29 ± 0.01 μm, mutant 0.28 ± 0.01 μm) were similar in both groups. However, control animals had two fold more synaptic vesicles per synapse, while mutants showed similar amounts as 2 days earlier (control: 9.8 ± 0.2 vesicles per synapse; mutant 5.0 ± 0.8; p=0.005). The amount of vesicles morphologically docked at the membrane was also doubled in control synapses, but similar to 2 days earlier in the mutant (control 2.1 ± 0.1; mutant 0.9 ± 0.5; p=0.080; Fig.3P-R). Thus, whereas the number of docked and total vesicles per synapse doubled in two days in the controls, the synapse morphology in the mutant was similar to two days earlier.

Synapses were less abundant in the mutant

In the marginal zone of the neocortex, the first morphologically identifiable synapses were reported at E15 (Konig et al., 1975). At E16, we observed few synapses in the control (8.5 ± 2.1 per 1000μm^2). The mutant

contained even fewer synapses (3.1 ± 0.5 per 1000μm^2; p=0.014), 3 fold less than controls (Fig.3S). Two days later, controls showed almost twice as many synapses, while mutants had similar values as 2 days earlier (control 15.5 ± 2.3; mutant 3.6 ± 1.4; p=0.005; Fig.3S). Thus, at E18, controls had almost 4 fold more synapses than mutants.

At both time points, approximately half the synapses contained morphologically docked synaptic vesicles. Similar to the quantification of all synapses (see above), we found that the abundance of synapses with docked vesicles almost doubled between E16 and E18 in controls, while the abundance of these synapses did not increase in the mutant. Consequently, synapses with docked vesicles were 3 fold more abundant in controls at E16 (control 5.1 ± 2.0; mutant 1.6 ± 0.5; p=0.060) and 5 fold at E18 (control 8.6 ± 1.4; mutant 1.7 ± 0.9; p=0.022; Fig.3T).

Thus at E16, controls and mutants had similar synapse morphology, but controls had 3 fold more synapses. Between E16 and E18, control synapses matured and increased in number, while the morphology and abundance of the mutant synapses remained the same.

The mutant marginal zone contained fewer "empty synapses"

We observed that the membranes of certain axons and/or dendrites were in contact by electron-dense material (denser than the rest of the membrane), but with no vesicles in the vicinity (Fig.4). High osmolarity Karnovsky fixative, which shrank the embryonic tissue (see Methods), revealed a protein smear between the two membranes (Fig.4C). This suggested that specific proteins were present in this specialization and may be involved in cell recognition or synapse formation. Such structures were also reported, for instance, in the rat embryo marginal zone and new-born visual cortex, and the pontine nuclei, as well as in the chick embryo optic tectum (Konig et al., 1975; Panzica and Viglietti-Panzica, 1980; Blue and Parnavelas, 1983a, b; Mihailoff and Bourell, 1986). These structures may either represent the first stage of synapse formation or a section through a part of the synapse that is devoid of vesicles. Here, these structures are referred to as "empty synapses". The lengths of the electron dense membranes were similar between controls and mutants (E16: control 0.230 ± 0.004μm; mutant 0.239 ± 0.008μm; E18: control 0.245 ± 0.012μm; mutant0.226 ± 0.014μm). However the empty synapse abundance was significantly different between the two groups. At E16, control animals had approximately 3 fold more empty synapses than mutants (control 35.3 ± 5.1; mutant 13.3 ± 1.65; p=0.002). The numbers of these structures decreased between E16 and E18 in both groups, but remained significantly different, controls having 3 fold more empty synapses than mutants (control 24.1 ± 2.6; mutant 8.1 ± 0.7; p=0.0004). Thus electron dense membrane specialization without vesicles, or empty synapses, were a normal feature during development, were more regularly found at E16 than E18 and mutants had significantly fewer.

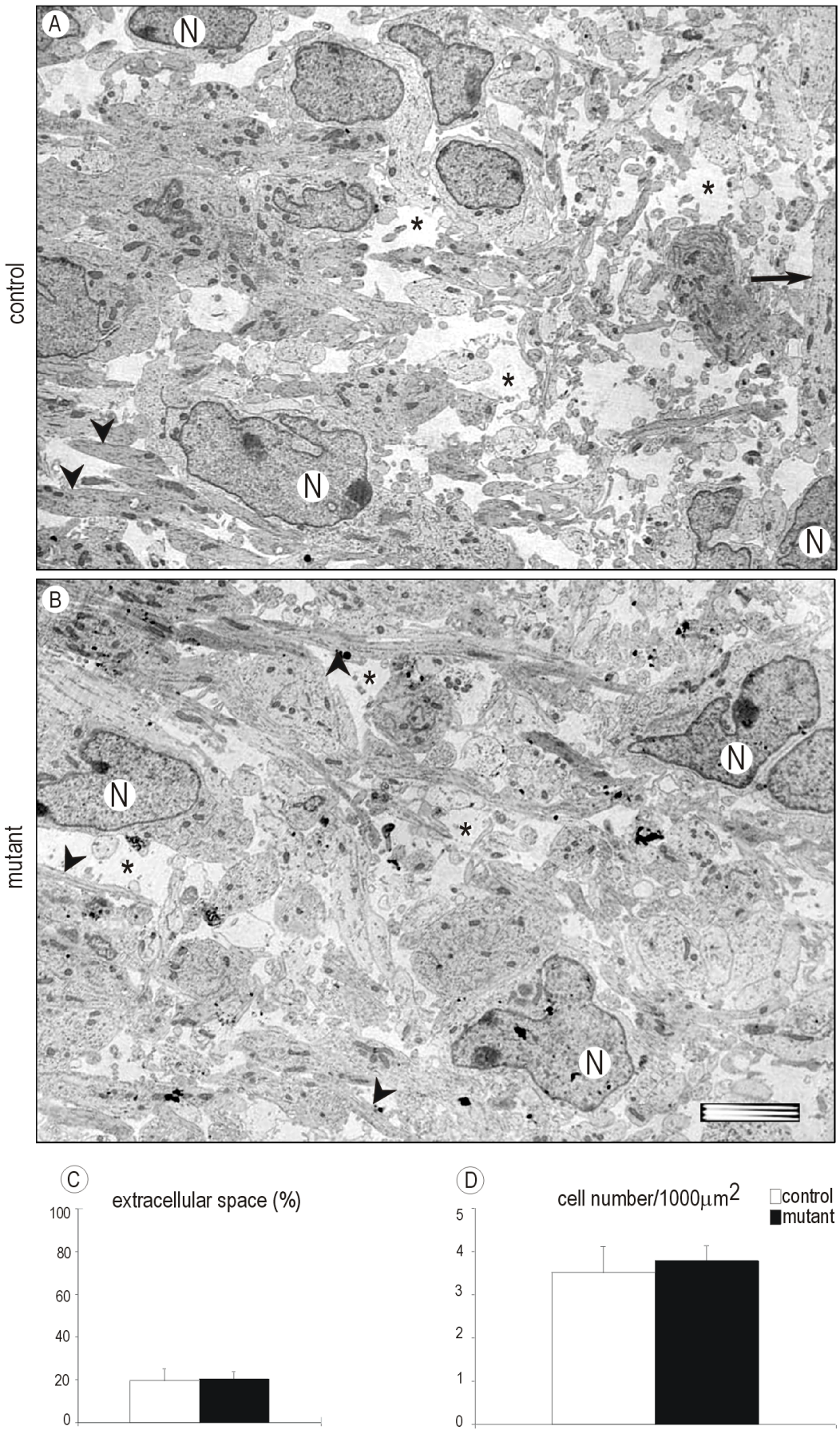

Fig.2) Low magnification photomicrographs showing overviews of coronal sections from neocortical marginal zone (layer I) of 18

day old normal (A) and munc 18-1 mutant (B) mouse embryos and morphometric results (C & D). Cortical plate neurones are to the left and pial to the right. **A**) Controls and **B**) mutants had few somata compared with the amount of axons (arrowheads) and dendrites (arrow). Immature neurones (N) were found scattered in the marginal zone. **C**) The percentages of extra cellular space (asterisk) were similar in both groups. **D**) The numbers of cells per 1000μm^2 of neocortical marginal zone were also similar in both groups. Bar: 6μm.

The mutant contained more "multivesicular structures" at E18

We also observed that the membranes of two axons and/or dendrites were in contact by electron dense material and both sides contained round and/or pleomorphic vesicles (Fig.5A & C). Such structures have been described in the developing brain, for instance in the marginal zone (Konig et al., 1975), in the olfactory lobe (Pinching and Powell, 1971) and in the pontine nuclei (Mihailoff and Bourell, 1986) and may reflect the elimination of a synaptic contact or the degeneration of the entire axon and/or dendrite, starting at the synapse. We refer to these structures as "multivesicular structures".

The length of the electron dense region of the membrane of the multivesicular structures was similar in controls and mutants (at E16: control 0.261 $\pm$ 0.006μm; mutant 0.264 $\pm$ 0.027μm; at E18: control 0.313 $\pm$ 0.025μm; mutant 0.276 $\pm$ 0.017μm). At E16, the abundance of multivesicular structures was similar in both groups (control 7.0 $\pm$ 1.9 per 1000μm^2; mutant 6.6 $\pm$ 2.5; P=0.789), but at E18 it was 2 fold more abundant in mutants (control 11.5 $\pm$ 3.5 per 1000μm^2; mutant 22.2 $\pm$ 2.55; p=0.051).

Vesicles and multivesicular structures stained for synaptic vesicle markers

To confirm the nature of the round and/or pleomorphic vesicles in the synapses and in the multivesicular structures, we performed immunogold labeling for two synaptic proteins on brain sections from mutants and controls at E18. In the synapses, VAMP/synaptobrevin (Fig.3D, I & N) and Rab3A (Fig.3E, J & O) were present at the vesicle membranes. In both groups, round and pleomorphic translucent vesicles were indiscriminately labeled with gold particles. Immunogold labeling also revealed that pleomorphic vesicles in the multivesicular structures contained these synaptic proteins (Fig. 4B & D). Thus, pleomorphic vesicles found in developing synapses and in multivesicular structures contained synaptic vesicle markers.

DISCUSSION

In the present study we quantified and characterized synapse formation and maintenance in the absence of spontaneous and evoked synaptic transmission using the munc18-1 mutant as a model. We found that the overall organization of the neocortical marginal zone was normal and that immature synapses had formed. Initially, the morphology of these synapses was similar to synapses in control littermates of the same age (E16), but controls had 3 fold more of these synapses. Between E16 and E18, control synapses matured and increased in number, while the morphology and abundance of the mutant synapses remained the same. Instead, the number of multivesicular structures increased in the mutants. Immunogold labeling re vealed that vesicles present in synapses and in multivesicular structures contained synaptic vesicle markers.

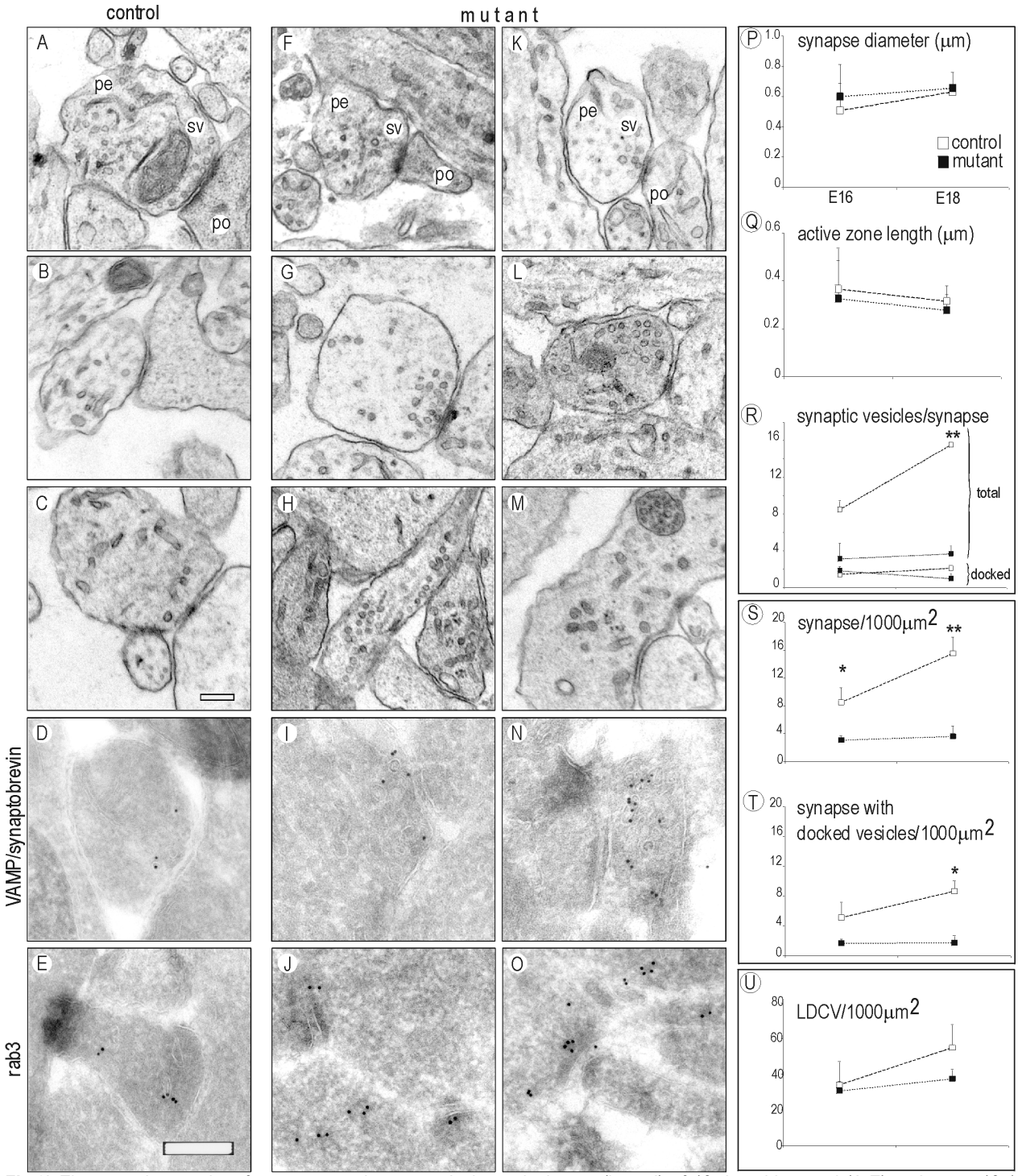

Fig.3) Electron micrographs of synapses at the neocortical marginal zone (layer I) of 18 day old normal (A-E) and munc 18-1 mutant (F-O) mouse embryos and synaptic morphometric results (P-U). **A-E**) Controls and **F-O**) mutants had similar developing synapses composed of a presynaptic element (pe) containing clear-round and/or clear-pleomorphic synaptic vesicles (sv), a postsynaptic (po) element and an electron dense membrane(arrow). **D, I & N**) Immunogold labelling of VAMP/synaptobrevin and **E**, **J** & **O**) rab3. This labelling revealed that membranes of round-clear and/or pleomorphic-clear synaptic vesicles were positive for those synaptic proteins. **P-R**) Synapse morphology. At embryonic day 16 (E16), controls and mutants had similar presynaptic morphology, i.e. presynaptic diameter, active zone length, total and docked synaptic vesicles. Two days later (E18), synapse morphology also did not differ, except for an increase in the total number of synaptic vesicles in controls without increased numbers of docked vesicles. **S**) Synapse abundance (number/unit area) of all synaptic profiles. At both time points, controls and mutants were significantly different. Control synapse abundance increased, while that of the mutant remained constant. **T**) Abundance of synapses containing at least one morphological docked synaptic vesicles. At E16, controls had 3 times more and at E18, 4 times more synapses than mutants. **U**) Large dense cored vesicle (LDCV) abundance was similar in both groups at both time points. However at E18, controls had more LDCV than mutants. ✲ = P<0.05, ✲ ✲ = P< 0.01. Bar: 200nm.

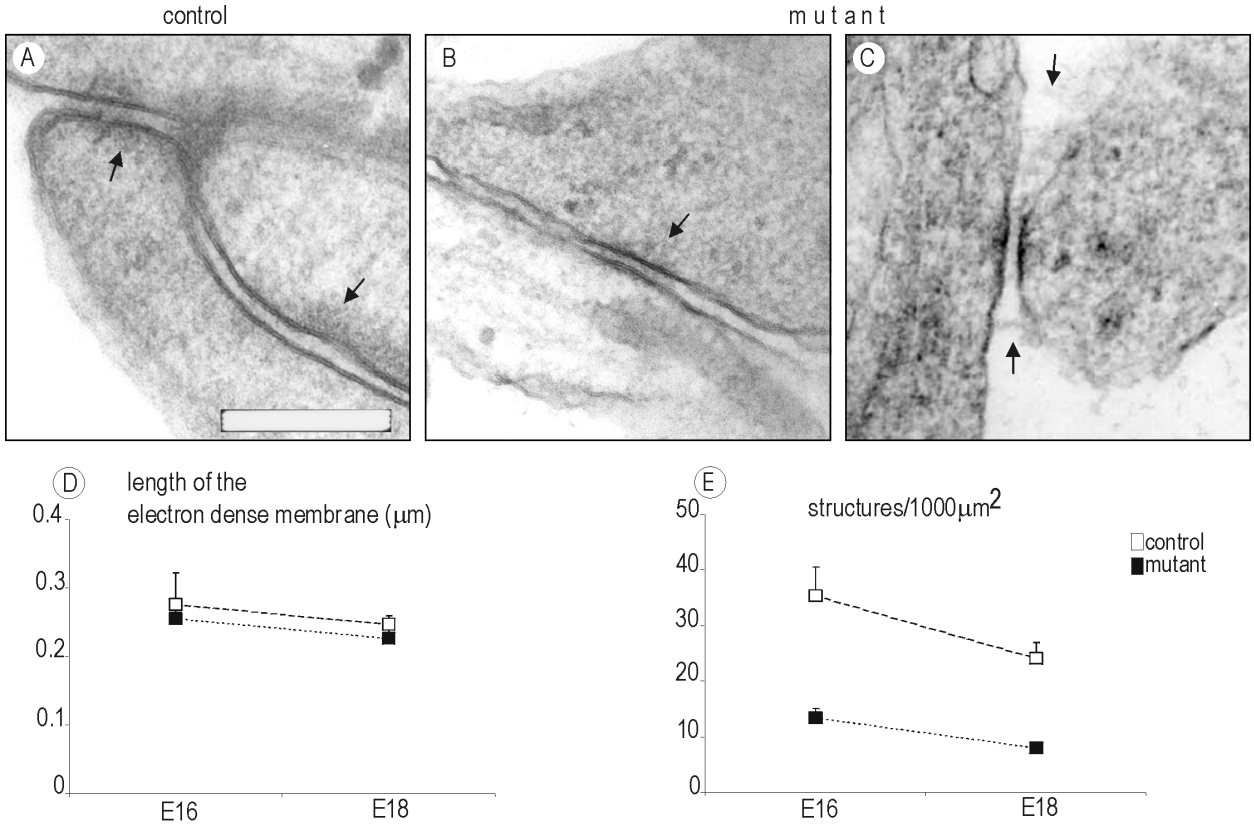

Fig.4) Electron micrographs of electron dense membrane, or desmosome-like, specialisation at the neocortical marginal zone (layer I) of 18 day old normal (A) and munc 18-1 mutant (B&C) mouse embryo and morphometric results (D&E). **A** & **B**) Electron dense membrane (arrow) between axons and/or dendrites in tri-aldehyde fixative and **C**) the same structure in Karnovsky fixative (see Methods and Results sections). Notice in C the smear of proteins (arrows) between the two cell processes. **D** & **E**) Empty synapse morphology. At E16 and at E18, controls and mutants had similar length of desmosome-like specialisation. However, desmosome-like abundance (number/unit area) was significantly different between controls and mutants at both time points analysed. ✲ ✲ = P< 0.01, ✲ ✲ ✲ = P< 0.001. Bar: 200nm.

In the munc18-1 mutant, massive degeneration takes place in lower brain regions like the brain stem between E16 and E18 (chapter 3). However, this is unlikely to explain the lower abundance of synapses in the neocortex of the mutants. Cortical plate neurons, which send axons to the marginal zone, do not receive and do not need extra-cortical innervation to differentiate at this developmental stage. Thus, all synapses found in the marginal zone are from cortical neurons. These neurons do not show signs of degeneration, also upon specific stainings for early markers of cell death/apoptosis (Maia et al., in preparation). Hence, we conclude that the lower abundance of synapses is not a consequence of neuronal degeneration in other brain areas. Conversely, the lower synapse abundance and failure to mature may be the primary defect that subsequently leads to degeneration. Generally, synapses in lower brain areas mature earlier in development than in the neocortex. It is conceivable that a lack of synapse maturation has already initiated cell death in the lower brain areas, whereas signs of degeneration are not yet detectable in the neocortex.

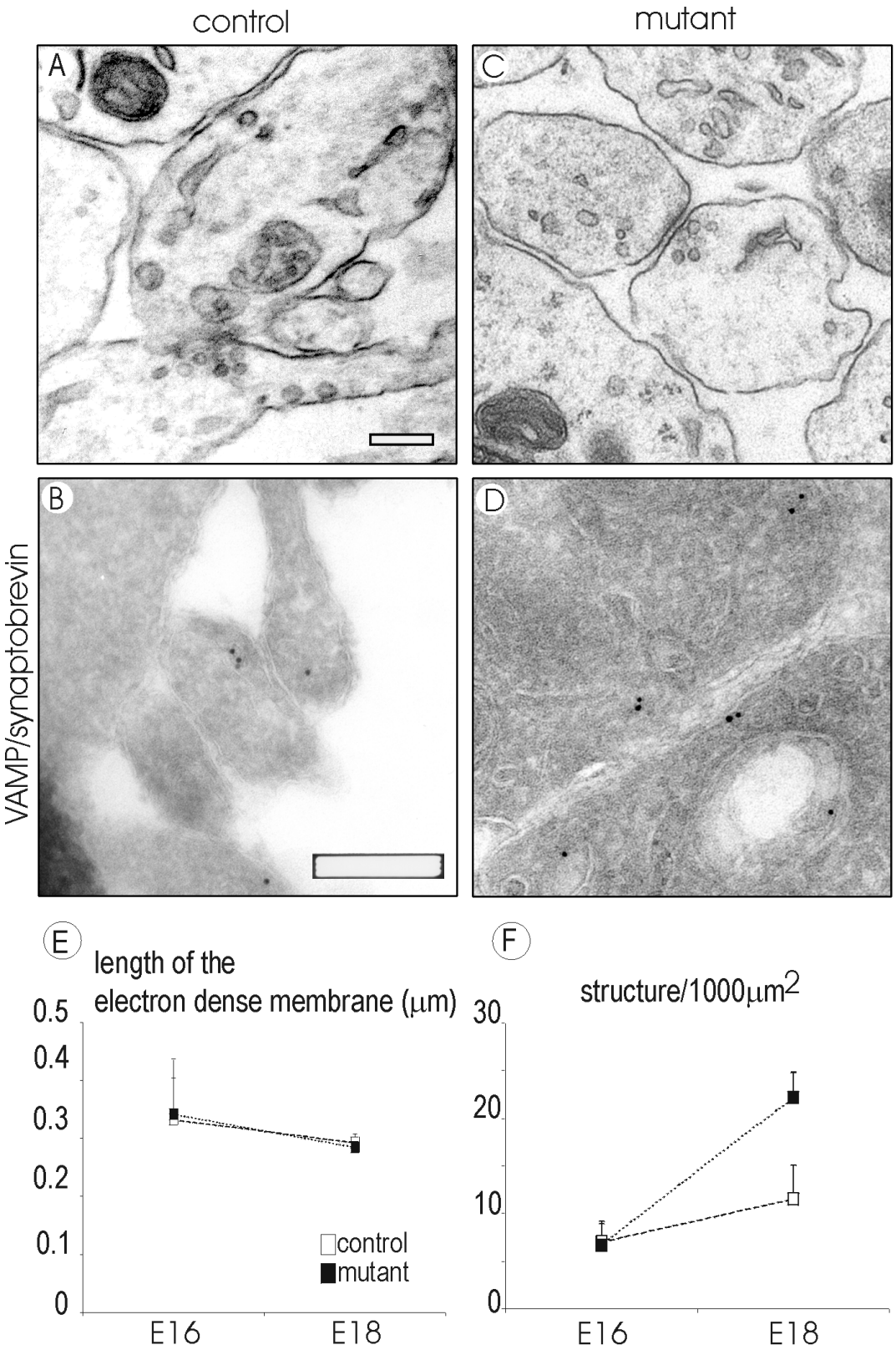

Fig.5) Electron micrographs of multivesicular structures at the neocortical marginal zone (layer I) of 18 day old normal (A & B) and null munc18-1 mutant (C&D) mouse embryo and morphometric results (E&F). **A** & **C**) Multivesicular structures, i.e. two cell processes containing round and/or pleomorphic vesicles (v) and joined by an electron dense membrane (arrow). **B** & **D**) Immunogold labelling of VAMP /synaptobrevin. Labelling was restricted to the membranes of round/pleomorphic vesicles and endosome-like organelles in both cell processes. **E** & **F**) Multivesicular structures morphology. At E16 and at E18, controls and mutants had similar electron dense membrane length and similar multivesicular structure density (number/unit area). However, at E18, mutants had the tendency to have more of those structures than controls. Bar: 200 nm.

A crucial difference between the neocortex with and without synaptic transmission is the observation that between E16 and E18 both the number of synapses and the number of vesicles per synapse do not increase in the absence of transmission, while these numbers are approximately doubled in the same period in controls. This observation may be explained by assuming that synaptogenesis is impaired in the absence of transmission and synapses stop maturing at a stage when approximately 5 vesicles (of which at least 1 was morphologically docked) have arrived in the terminal. However, developing synapses are highly plastic, i.e., undergo rapid changes (see for instance Haydon, 1988; Meinertzhagen, 1993). It is not plausible that the immature synapses in the mutant remained static for two days. Furthermore, in the same period, between E16 and E18, the number of multivesicular structures increased in the mutant. In fact, the absolute difference in synapses and in multivesicular structures is approximately equal. This observation also argues against a simply delayed development in the mutant. Thus, the most likely explanation is that synapses observed at E16 in the mutant transformed to multivesicular structures observed at E18 and that the synapses observed at E18 are novel synapses. This suggests that synaptogenesis is unimpaired in the absence of transmission and that immature synapses with a few synaptic vesicles mature further only when the synapse becomes active and transforms to a multivesicular structure when it remains silent.

Multivesicular structures are found in normal developing brains, but >2 fold more often in the mutant. These structures may represent the first step in synapse disassembly. It is still a matter of debate how CNS synapses are eliminated (see Wolff et al., 1995; Bernstein and Lichtman, 1999), but the fact that the vesicles in these structures are labeled for synaptic vesicle proteins suggests a synaptic origin and the fact that they increase in abundance in the mutant when synapses become more abundant in a normal developing brain suggests that these structures may indeed represent disassembling synapses. These multivesicular structures may be the first morphological sign of a cascade of events that ultimately leads to the disassembly of whole brain areas as observed in lower brain areas of the mutant (chapter 3). Interestingly, elimination of a neuromuscular synapse depended on the activity of strong synapses, producing ‘punishment signals’ to weaker synapses (see for a review Lichtman and Colman, 2000). Since the munc18-1 deficient nervous system is characterized by a complete absence of transmission, the synaptic disassembly observed in these brains is probably accounted for by a different mechanism. It is also plausible that this disassembly and the appearance of abundant multivesicular structures is initiated from the somata, which are devoid of input. It was previously shown that after silencing neuronal networks in culture, the first signs of cell death are observed at the synapses (Williamson and Neale, 1998).

Several recent reports describe other mutant mice that lack certain aspects of vesicular secretion and also mutants that lack important receptors for the secreted transmitters (Geppert et al., 1994a; Augustin et al., 1999). However, abundant multivesicular structures are not reported for any of these. Disassembly of brain areas is not observed either. This may be explained by an additional defect in the munc18-1 mutant intrinsic to the deletion of the gene rather than the result of blocking transmission. However, two preliminary findings (Heeroma, Van Aerde and Verhage, in preparation) argue against this explanation. First, primary sensory neurons in the mutant are probably the only neurons with normal input/activity, since this does not depend on transmitter secretion. These neurons, for instance in the dorsal root ganglia, mature relatively early in development, but do not show degeneration. Second, massive cell death is also observed in neuronal cultures from mutant brains. This cell death can be rescued by co-culturing these neurons with wild type neurons. This suggests that at least cell death and probably also the lack of synapse maturation can be explained by the lack of activity and that an intrinsic defect in the neurons caused by munc18-1 deletion is not a major cause.

No other published gene deletion is characterized by a complete and permanent loss of all aspects of vesicular secretion, evoked and spontaneous, in all transmitter systems. It is conceivable that this difference explains the apparent variation in the integrity of the neuronal networks between munc18-1 and other models. Hence, at the early stages of synaptogenesis, when the first contact is formed and a few synaptic vesicles have arrived in the terminal, low levels of activity are sufficient to prevent loss of synapses. Probably only the spontaneous, infrequent fusion of synaptic vesicles is sufficient. Two previous animal models may also completely lack synaptic transmission (including spontaneous events) in some synapses: zebrafish lacking acetylcholine receptors (Westerfield et al., 1990) and GABA-deficient worms (Jin et al., 1999). Still both animals made synapses and in the case of GABA-deficient worms, the synaptic connectivity of the nervous system was approximately normal and no degeneration was observed. Hence, these two models argue against an essential role for spontaneous transmitter release in stabilizing neuronal networks. However, in both models, transmission itself was not assayed. Possibly, other transmitters or receptors account for some residual activity that may rescue synapses, because secretion itself was not blocked in these models. Furthermore, synaptogenesis is probably not regulated in the same manner in fish/worms and mammals, because acetylcholine-deficient mice, unlike acetylcholine receptor deficient fish, have no stable neuromuscular synapses (Brandon et al., 2000). Finally, preliminary data suggest that deletion of another essential component of the secretion machinery, SNAP25, leads to similar degeneration, at least in neuronal cultures, as observed *in vivo* for munc18-1 deletion (Washbourne et al., 2000).

Cell differentiation and survival in the developing mouse brain in the absence of synaptic activity

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ABSTRACT

In vitro experiments suggest that neurones require active input to survive. The role of synaptic activity for survival *in vivo* in less well understood. In the homozygous munc 18-1 null mutant mouse, synapses are not functional. Using mutant embryos, we investigated neuronal and glial differentiation. We found that certain neurone groups were fully differentiated (the neocortex); some were partially differentiated (hippocampus, most areas of the thalamus and hypothalamus) and others were degenerated or degenerating (mitral cell layer of the olfactory lobe, certain thalamic and hypothalamic nuclei, cerebellum, tectum, medulla and spinal cord) suggesting that early maturing areas degenerate first. In the degenerating areas, massive apoptotic cells were observed, which induced massive macrophage activation. Gliogenesis occurred prematurely in some foci (hippocampus, hypothalamus and medulla). Our results suggest that synaptic activity is essential for completing cell differentiation. In the absence of this activity, cell differentiation is disturbed and neurones die by apoptosis.

INTRODUCTION

Central nervous system (CNS) development involves a cascade of morphologically distinguishable events such as neurulation, cell differentiation, cell migration, axon outgrowth, synapse formation and apoptotic cell death and gliogenesis. Specific genes are switched on and off along this cascade of events and serve as landmarks for these developmental steps, e.g. during glia differentiation, the glial fibrillary acidic protein (GFAP) identifies mature astrocytes (Bignami et al., 1972).

The timing of these developmental events is not the same for all CNS cells. Furthermore, these events are not mutually exclusive, they can happen simultaneously to the same cell type. For instance before birth in the embryonic olfactory epithelium, the olfactory receptor neurones differentiate among the epithelial cells and do not migrate. Shortly after differentiating, they grow axons reaching the presumptive olfactory lobe by embryonic day 12 (E12) and influence olfactory lobe formation (Gong and Shipley, 1995). Meanwhile cell death has occurred since the beginning of the olfactory epithelium development and continues postnatally (Voyron et al., 1999). In adults, the olfactory receptor neurones make synapses on tufted and mitral cell dendrites of the olfactory lobe (Pinching and Powell, 1971). In the embryonic olfactory lobe, differentiating neurones show their first synapse at E14 (Hinds and Hinds, 1976a, b). One day later, few morphologically differentiated neurones are recognisable as mitral cells and have already started growing axons, i.e. the lateral olfactory tract (Hinds, 1972). In this system, it is unknown whether spontaneous synaptic activity from the olfactory receptor neurones induces mitral cell differentiation.

Mouse spinal cord neurones acquire differentiated specialisation, i.e. synapse appearance already by E11 (Vaughn, 1989), while morphologically identifiable astrocytes are only visible perinatally (Goldman and Vaysse, 1991). Apoptotic cell death of superfluous spinal cord neurones happens in foci in a time-dependent manner, starting at E11 (Yamamoto and Henderson, 1999). Furthermore, in new-born rat spinal cord, there is evidence that growing axons and immature glial cells establish synapse-like junctions (Gorgels, 1991). Yet again, it is unknown whether spontaneous synaptic activity plays a role on this glial differentiation delay and/or in the spinal cord neuronal elimination.

The aim of the present study was to investigate the effect of eliminating spontaneous synaptic activity on cell differentiation and survival. For this purpose we used the 18-1 mutant mouse in which synaptic activity (spontaneous and evoked) is abolished (chapter 3). We found that, compared with the controls, mutants' brains had normal differentiated cells (e.g. the neocortex), partially differentiated brain areas (e.g. hippocampus, thalamus and hypothalamus), degenerating or degenerated neurones (e.g. mitral cells, cerebellum, medulla and spinal cord). Gliogenesis was premature in some foci. Cell degeneration was apoptosis and this induced massive macrophage activation.

METHODS

Animals

Generation of germline-transmitting munc 18-1-deficient mice and PCR genotyping are described in chapter 2 and 3. Briefly, exons 2 to 5 of the munc 18-1 gene were replaced with a neomycin resistance gene using homologous recombination. The mouse colony was maintained by breeding of heterozygous animals. Heterozygous males were mated with synchronised heterozygous females for one night. If a

vaginal plug was detected the following morning, this was considered embryonic day (E) zero. Embryos were harvested from mothers rapidly sacrificed after timed matings at E18. Embryo heads were used for electron microscopy and a posterior paw was sampled for genotype analysis. Experimental procedures were carried out with local regulatory approval for animal experimentation.

Anatomical analysis

Mouse embryos at 18 days of gestation were fixed by immersion in Bouin fixative (71.4% saturated picric acid, 23.8% formalin and 4.8% acetic acid) for 72h at room temperature. After dehydration they were embedded in paraffin. Coronal and sagittal sections (5μm) were cut, mounted on gelatine-coated glass slides and dried overnight at 45°C. They were stained with hematoxylin/eosin cresyl violet for anatomical analysis. Brain anatomical identification and nomenclature here used are based on the "Atlas of the prenatal mouse brain" (see Schambra et al., 1992).

TUNEL staining

Embryo sections were prepared as described above. We followed a protocol described by van der Salm et al. (2000). Briefly, sections were deparaffinized, rehydrated and microwaved in 0.1% Triton-Na-citrate buffer in 10% PBS. Apoptotic cells were labelled using the cell death detection kit, fluorescein (Boehringer Mannheim cat.no.1684795). To visualise the fluorescein, peroxidase-conjugated converter enzyme POD (cat.no.1684817, Boehringer Mannheim) was utilised and this was stained with 3',3'-diaminobenzidine (DAB)-Ni. Negative controls were included by omission of either the TUNEL enzyme or the POD converter enzyme.

Electron microscopy

E18 mouse embryos were decapitated. In order to give the fixative fast access to the brain, a few drops of tri-aldehyde fixative (2,5% DMSO, 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolin in 0.1M NaCacodylate pH7.4) were poured onto the heads and skin and skull were peeled off. The entire head was cut sagittally between the two hemispheres. In this state, the heads were put in tri-aldehyde fixative for 1h at room temperature and then overnight at 4°C. The next day, further dissection was carried out dividing the brain in small pieces. Afterwards, we followed a protocol described elsewhere (Gorgels, 1991). Briefly, the small pieces were post fixed for 2h at room temperature in OsO_4 2%-$KRuCN_6$ 4% in cacodylate buffer 0.4M, dehydrated and embedded in EPON. Ultrathin sections were then mounted on non-coated grid and contrasted with uranyl acetate/lead citrate.

Immunohistochemistry

F4/80 staining. Embryos were decapitated and immediately frozen in dried ice. Cryosections (10μm) were cut, mounted on gelatine-coated glass slides and dried overnight in a humidproof box. Sections were fixed for 10min in pure acetone and air dried, then incubated with rat monoclonal antibody (Ustyn and Gordon, 1981)/1% bovine serum albumin (BSA) for 1h, washed 3 times and incubated for 1h with a biotinylated rabbit anti rat antibody (DAKO, Denmark). The whole procedure was performed at room temperature and the buffer for all steps was 10mM PBS.

GFAP staining. Paraffin sections were rehydrated and microwaved (640W) 3 times 5 min in 0,1% Froggy commercial detergent (15-30% anionogenic, <5% ionogenic and amphoteric surfactants; Werner and Mertz, Waterloo, Belgium). After each microwave treatment, they were allowed to cool and initial volume was restored with demi water. Endogenous peroxidase was blocked with 0.6% H_2O_2 in 100% methanol for 30min. Sections were incubated overnight in a humid chamber with rabbit GFAP antibody (DAKO Denmark, cat.no.Z0334)/1% BSA. After washing 3 times they were incubated for 2h with a biotinylated goat anti rabbit antibody (Vector Lab, cat.no.BA-1000)/1% BSA. Except for the microwave, the whole procedure was executed at room temperature. Tris buffer saline (tbs) (50mM) buffer was used in all steps, except when explicitly stated.

Sections were incubated with avidin biotin complex (Vectastain ABC Kit, Brunschnig, Amsterdam) for 1h after both biotinylated antibodies, washed three times and incubated with DAB in 50mM Tris/HCl pH7,6 buffer. The reaction was stopped when colouring was satisfactory.

RESULTS

Without synaptic activity, the brain is a patchwork of differentiated, partially differentiated and degenerated cells

Anatomical analysis showed that mutant embryos developed all body parts (organs and tissues), such as the eye (E), tongue (T), pituitary (P) and the trigeminal ganglion (TGG) (Fig.1 A & G). Munc18-deficient mice contained one brain area with normal morphology, whereas other regions were abnormal (see below).

Normally differentiated cells. We have previously shown that the control and mutant neocortex (NC) were similarly layered. In both cases, the neocortex was composed of mitotic cells in the subventricular zone, migrating neurones in the cortical plate and growing axons in the white matter and marginal zone layer (Fig.1B-E, H-K; chapter 3 and 4).

Thus, the mutant visual system was not degenerated, i.e. the eye (E), optic nerve (ON), optic chiasma (OC) and the future visual cortex (NC) were normally constituted (Fig.1A-E, G-K).

Partially differentiated cells. The control hippocampus (H) had a well-differentiated pyramidal cell layer and differentiating granule cells of the dentate gyrus (Fig.1E). Although the mutant hippocampus was clearly identifiable and cells were present at the place of the pyramidal cell layer, these cells did not complete their migration like in controls. (Fig.1K). Control thalamus and hypothalamus had clear thalamic nuclei (e.g. the periventricular nucleus (PVT) and the medial thalamic nucleus (MT)) as well as clear hypothalamic nuclei (e.g. anterior hypothalamus (AH) and the premammillary nucleus (PRM)) (Fig.1D & E). In contrast in mutants, cells assembled in those areas but did not form these nuclei (Fig.1J & K).

Degenerated and/or degenerating cells. In the normal olfactory system, a group of immature neurones in the olfactory lobe differentiate into the mitral cells. They acquire a perpendicular orientation to the olfactory lobe surface. These cells align themselves, form the mitral cell layer (MC) (Fig.1A), and grow axons, i.e. the lateral olfactory tract (LOT) (Fig.1B-D) (Hinds, 1972; Hinds and Ruffett, 1973). Although mutant olfactory receptor neurones differentiated and innervated the olfactory lobe (not shown), immature neurones did not differentiate into the mitral cells (Fig.1G) and consequently, the lateral olfactory tract was not formed (Fig.1H-J). Further, the mutant olfactory lobe was composed of an immature homogenous cell population around the olfactory lobe ventricle and was smaller than the control (Fig.1G). In normal animals before birth,

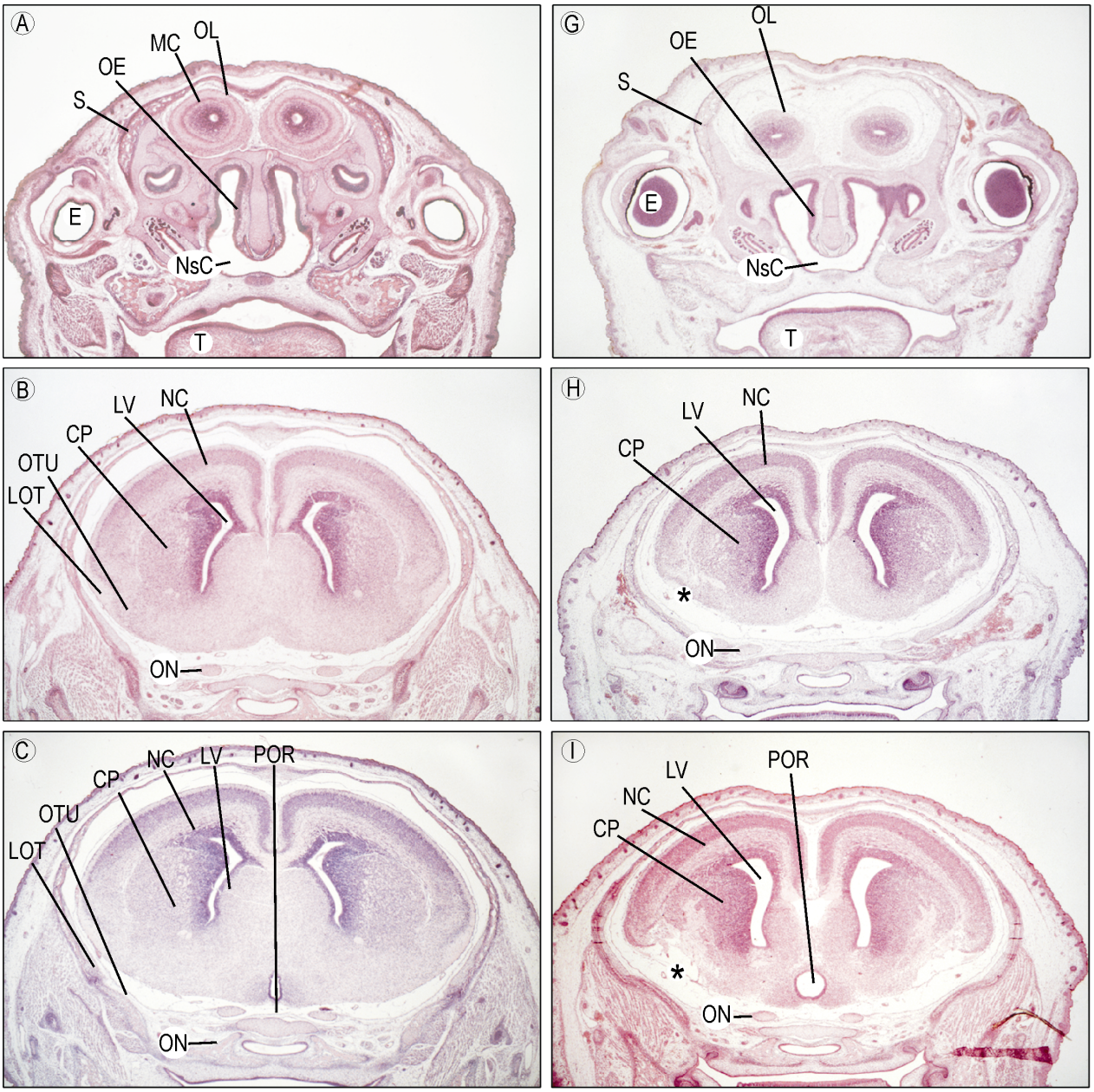
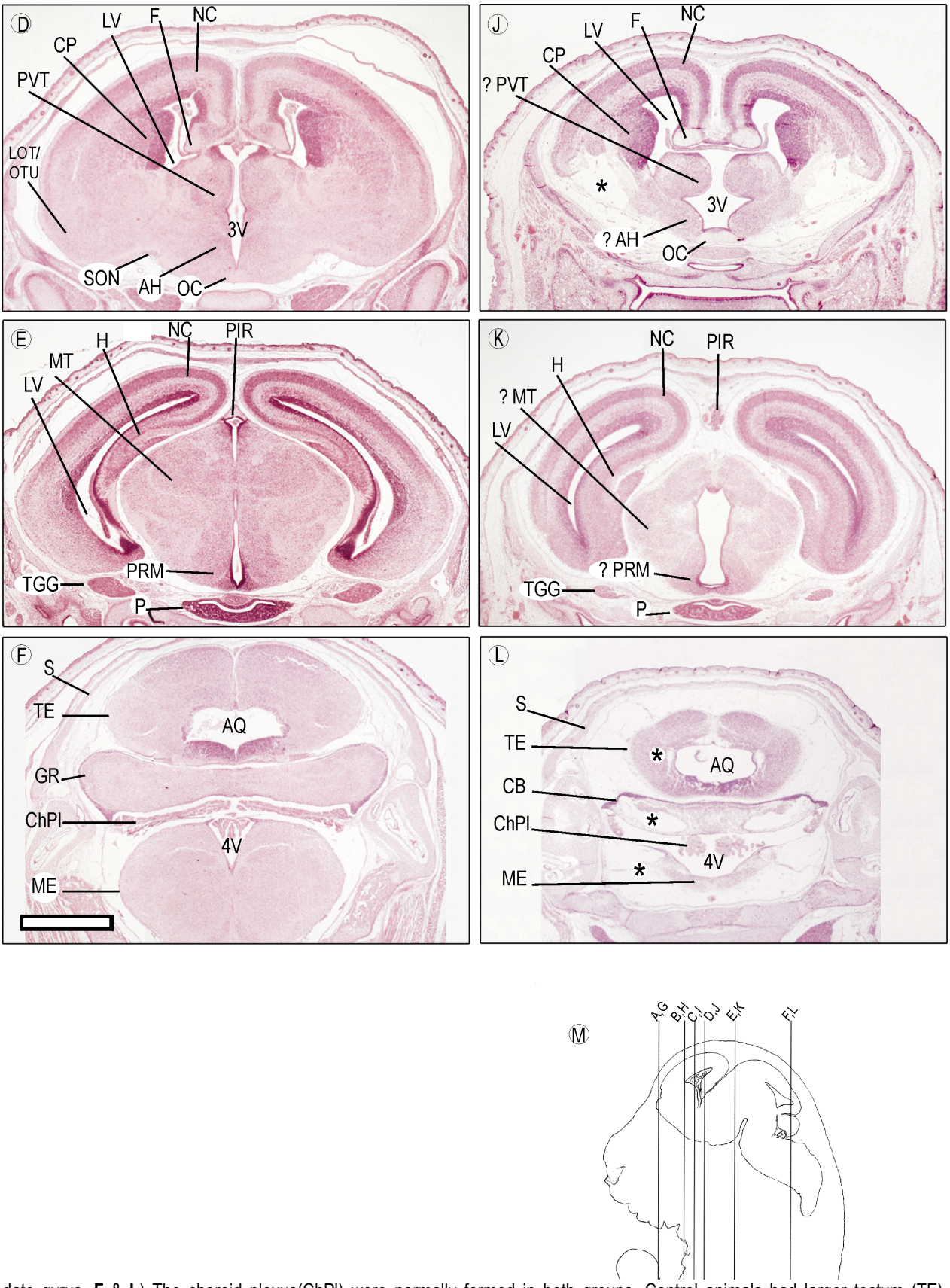


Fig.1) Low magnification micrographs of hematoxylin-eosin stained coronal sections of 18-day-old wild type (A-F) and munc 18-1 mutant (G-L) mouse sibling embryos. **A** & **G**) Both groups developed all organs and tissues, in particular notice the eyes (E) and the olfactory epithelium (OE). However, the mutants' brains differed from those of the controls. Controls had a distinguishable differentiated mitral cell (MC) layer of the olfactory lobe (OL). Mutants had a smaller olfactory lobe, than those of the controls, and no differentiated mitral cell layer. Notice in the mutants, the gap between the olfactory lobe and the skull (S). **B** & **H**) Control animals had a distinct caudate putamen (CP) and lateral olfactory tract (LOT), as well as a layered neocortex (NC). Mutants had no lateral olfactory tract but a normal differentiated neocortex. Notice the optic nerve (ON) in both groups. **C** & **I**) In controls, the olfactory tubercle (OTU) was identifiable. Here, mutants had bilateral gaps of missing cells (asterisk), slightly reaching the caudate putamen. The mutant lateral ventricle (LV) and preoptic recess (POR) were larger than those of the controls. **D** & **J**) In both groups, fimbrae (F) and optic chiasma (OC) were normally developed. Controls had a discernible periventricular thalamic nucleus (PVT) and anterior hypothalamus (AH), which were partially differentiated in the mutants (?PVT; ?AH). Further, the mutants' third (3V) and lateral ventricle were larger than those of controls. The asterisk indicates missing cells. **E** & **K**) The pituitary (P), trigeminal ganglion (TGG) and pineal gland or recess (PIR) were normal in both groups. Controls had recognisable thalamic (e.g. the medial thalamic nucleus (MT)) and hypothalamic nuclei (e.g. the premammillary nucleus (PRM)). Control hippocampus had an unmistakable pyramidal cell layer and developing granule cells of the dentate gyrus. While in mutants, cells were forming large abnormal brain nuclei, such as the cells at the presumptive medial thalamic nucleus (?MT) and premammillary nucleus (?PRM). Again, mutant hippocampus accumulated a partially differentiated pyramidal cells and granular cells of the den-



date gyrus. **F** & **L**) The choroid plexus(ChPl) were normally formed in both groups. Control animals had larger tectum (TE), cerebellum (CB) and medulla (ME) than mutants. Mutants were missing cells (asterisks) on those areas but some were still present. The mutants' forth ventricle (4V) was larger than that of controls. Notice the gap between the brain and the skull (S) in mutants. **M**) Schematic sagittal section indicating where the coronal sections were made. The letters indicate the labelled photos. Bar: 1000μm.

caudate putamen efferent neurones receive innervations before acquiring full morphology and physiological function (Voorn et al., 1988; Parent and Hazrati, 1995). In control animals the substantia nigra, one of the caudate putamen afferents, was normally developed (not shown) and the caudate putamen was clearly defined with differentiating neurones (Fig.1B-D). Although the mutant substantia nigra started developing (not shown), the dorsal caudate putamen was degenerated (Fig.1H-J). The control cerebellum had no circumvolutions but the granular cell layer (GR) was clearly discernible (Fig.1F). In contrast, the mutant cerebellum contained homogenous undifferentiated cells and a bilateral gap of missing cells (Fig.1L). The control medulla was composed of fibres and brain nuclei. However, the mutant medullar was greatly degenerated and had few cells and fibres. The remaining neurones were bordering the forth ventricle (4V) (Fig.1L).

Thus cells that normally receive synaptic input, like the mitral cells, in mutants could not proceed with their differentiation and were degenerated or degenerating

Degeneration of the mutant brain occurs through massive apoptosis

Apoptotic cell death is a normal event during development (for review see Naruse and Keino, 1995). It is characterised by DNA fragmentation, cell condensation, leading to cell shrinkage, and cell partitioning into apoptotic bodies. A major characteristic of apoptosis is that there is no cell leakage, and no inflammatory reaction. In control brains, condensed cell nuclei, suggesting apoptosis, were rarely encountered. However, this was frequently seen throughout the mutant brains (Fig.2). To confirm that the condensed cell nuclei were caused by apoptosis, we performed nick end labelling of the fragmented DNA (TUNEL technique) and electron microscopical investigation.

The condensed cell nuclei seen in the anatomical preparations were stained by TUNEL. In controls this was rare (not shown) and in mutants there were a higher number (Fig.3). In the anterior forebrain, apoptotic $TUNEL^{+}$ cell nuclei occurred at the ventral side but not in the dorsal side, i.e. the neocortex (Fig.3A&D). In the hippocampus, few cells underwent apoptosis in the putative pyramidal, moleculare and radiatum layers and no apoptosis was observed in the dentate gyrus (Fig.3B). In the mutant thalamus, apoptotic cells occurred at the periphery of the presumptive medial thalamic nucleus (MT). The presumptive anterior hypothalamus (AH) was filled with apoptotic cells (Fig.3C). At high magnification, single apoptotic bodies were observed either singly free in the tissue or in clusters (Fig.3D-F).

Electron microscopy results confirmed the TUNEL staining and revealed early apoptotic stages, which are not stained by TUNEL. Among thalamic immature neurones, there were apoptotic cells at distinct stages of apoptosis. These cells were electron denser (nucleus and cytoplasm) than neighbouring cells (Fig.4A-C). They had a dilated nuclear envelope lumen, cytoplasmic vacuoles and degenerating mitochondria. They shrunk while keeping contact with neighbouring cells. Apoptotic bodies were detached from the neighbouring cells and contained only cellular debris (Fig.4D-G).

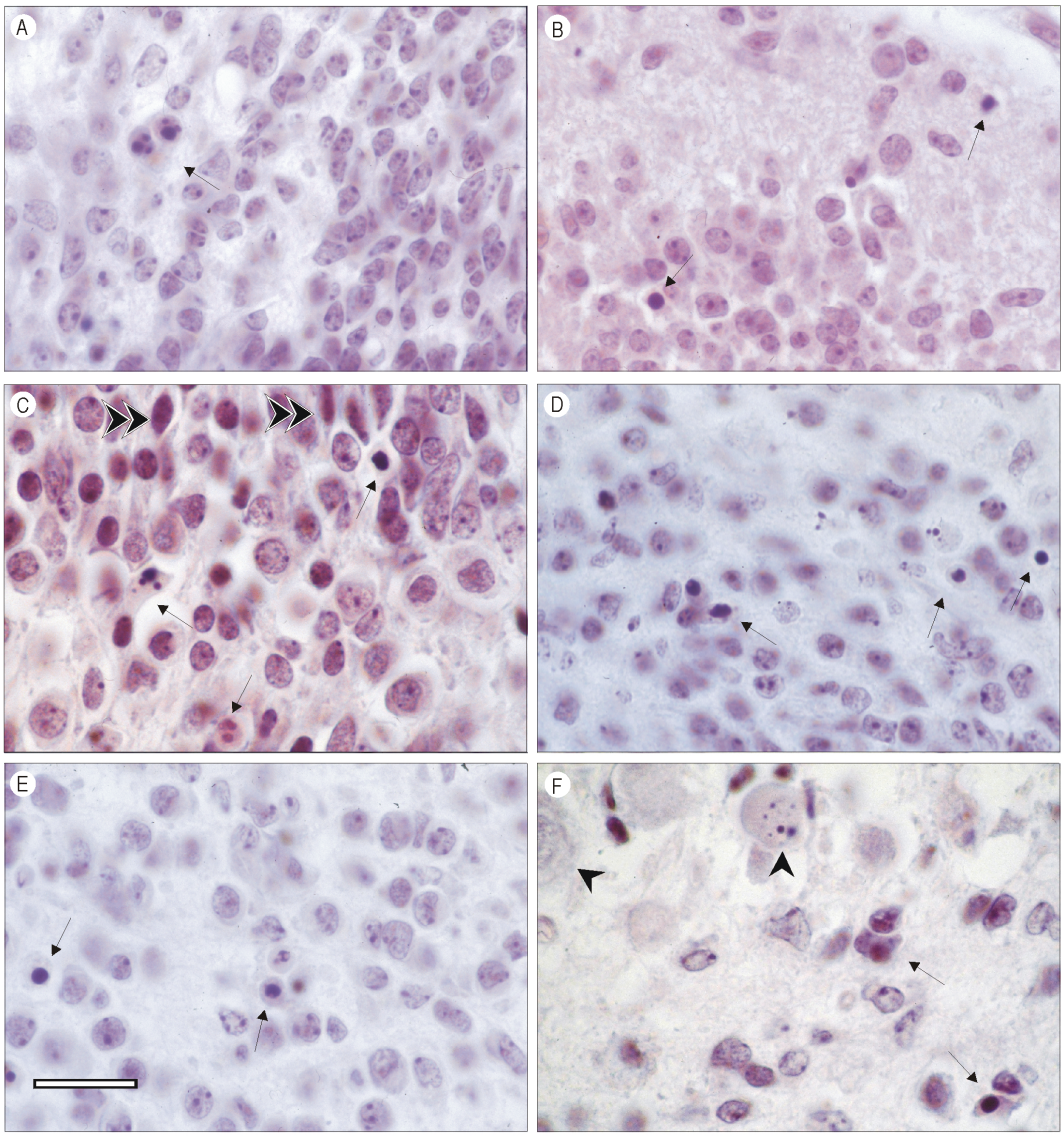


Fig.2) High magnification micrographs of hematoxylin-eosin stained sagittal sections of 18-day-old munc 18-1 mutant (A-F) mouse embryos. Arrows indicate condensed cell nuclei (apoptotic bodies) in the (**A**) olfactory lobe; (**B**) anterior forebrain; (**C**) hippocampus; (**D**) thalamus and (**E**) hypothalamus. In the (**F**) medulla, in addi-tion to the putative apoptotic bodies, there were still larger neurones (arrowheads). Bar: 20μm.

Massive macrophage activation in mutants

After cell death, apoptotic bodies are scavenged by macrophages. To verify whether macrophages were activated in the mutant embryos, we performed an immunostaining for the macrophage plasma membrane protein F4/80 (Fig.6) (Ustyn and Gordon, 1981; Lawson et al., 1992).

Control F4/80+ cells were seldom found in the brain tissue, but frequently in ventricles, intermingled with the choroid plexus, between the brain and the pia mater, or between the tectum and the cerebellum. The little staining that was found in the control brain tissue occurred in subventricular zone (SVZ) and tectum (Fig.5A-D). By contrast in the mutant, activated macrophages were observed throughout the brain tissue, as well as outside of the brain (Fig.5E-H). The staining clearly demarcated cell membranes, revealing some macrophages containing condensed cell nuclei (Fig.5I-L). Furthermore, phagocytes were also confirmed by electron microscopy at different stages of activation (Fig.6).

Neurone absence allowed premature gliogenesis in some foci

Since several neuronal populations were not morphologically identified, we studied the mutant brain for the glial marker GFAP. Mature astrocytes (GFAP+ cells) were found in the hippocampus, hypothalamus, medulla and spinal cord (Fig.7).

The control hippocampus had only faint GFAP+ dendrites in the oriens layer (Fig.7B). However, the mutant hippocampus had GFAP+ cells scattered in the dentate gyrus, in the fimbrae and in the oriens layer (Fig.7F). Control thalamus, hypothalamus and optic chiasma were free of GFAP+ cell, except at the periphery of the ventral hypothalamus where few dendrites were found (Fig.7C). The mutant thalamus was also unstained, except by a few positive dendrites bordering the lateral ventricle (Fig.7G). However in the mutant hypothalamus, GFAP+ cells were abundantly throughout the tissue. Mutant optic chiasma fibres were surrounded by astrocytes resembling the staining pattern in the fimbrae fibres (Fig.7G).

While control animals rarely had GFAP+ cells in the medulla/spinal cord, mutants had very many mature astrocytes. These were not randomly scattered, but occurred in a specific pattern, i.e. parallel or perpendicular to the axonal fibres (see chapter 2). The perpendicular dendrites were also established contact with the forth ventricle and/or mesencephalic flexure. Further the astrocyte staining pattern resembled the postnatal situation (Landry et al., 1990; Goldman and Vaysse, 1991; Astic et al., 1998). The cerebellum and tectum were devoid of astrocytes.

DISCUSSION

Synaptic vesicles and plasma membranes cannot fuse in munc 18-1 null mice (chapter 2 and 3). Using 18-day-old embryos, we investigated whether neurones and glia cells could complete their differentiation and survive without synaptic activity. Compared with controls, some mutant neurones were similarly differentiated, others were partially differentiated and others were degenerated and/or degenerating. Degeneration happened by massive apoptotic cell death, which induced massive macrophage activation. Astrocyte differentiation occurred prematurely in some foci, whose staining pattern resembled the postnatal situation and partially those of the macrophages.

Why are the munc18-1 neurones dying? It was suggested that the munc 18-1 protein is necessary for cell survival because overexpression of syntaxin, a munc 18-1 binding partner, in pc12 cells caused Golgi and endoplasmic reticulum deformation and consequently cell death. This was circumvented when munc 18-1 was transfected together with syntaxin (Rowe et al., 1999). However in contrast, the absence of munc 18-1 protein in mutants *in vivo* caused a decrease in syntaxin protein levels (Maia et al., chapter 2) and no cell deformation (chapter 4). Therefore *in vivo* munc18-1 is not necessary for preserving normal organelle structure. Supporting our previous results, here we showed that mutant differentiating thalamic neurones, without developed Golgi or endoplasmic reticulum, show no cellular deformations and die by apoptosis (Fig.4). The cause of cell death is plausibly explained by the lack of functional synaptic activity. First, in munc18-1 mutants, sensory neurones which normally do not receive synaptic innervation, are not degenerated, e.g. olfactory receptor neurones (Fig.1A&G) and dorsal root ganglion neurones (not shown). Second, the areas first forming synapses degenerated. Third, Ikonomidou and colleagues (1999) showed that *in vivo*, transient blockage of NMDA receptors, during the period of NMDA receptor hypersensitivity, triggers a wave of apoptotic neurodegeneration. Together these results suggest that *in vivo* spontaneous synaptic activity is the means by which neurones and glia (see below) co-ordinate their differentiation and their decision for survival or death.

Glia cells are not simply neurone supporting cells (Bergles and Jahr, 1998; Araque et al., 1999; Ventura and Harris, 1999). Neurone and glia interact morphologically via neuronal synapse-like structure (Gorgels, 1991) and physiologically controlling synapse numbers (Nelson et al., 1995; Ullian et al., 2001). So why

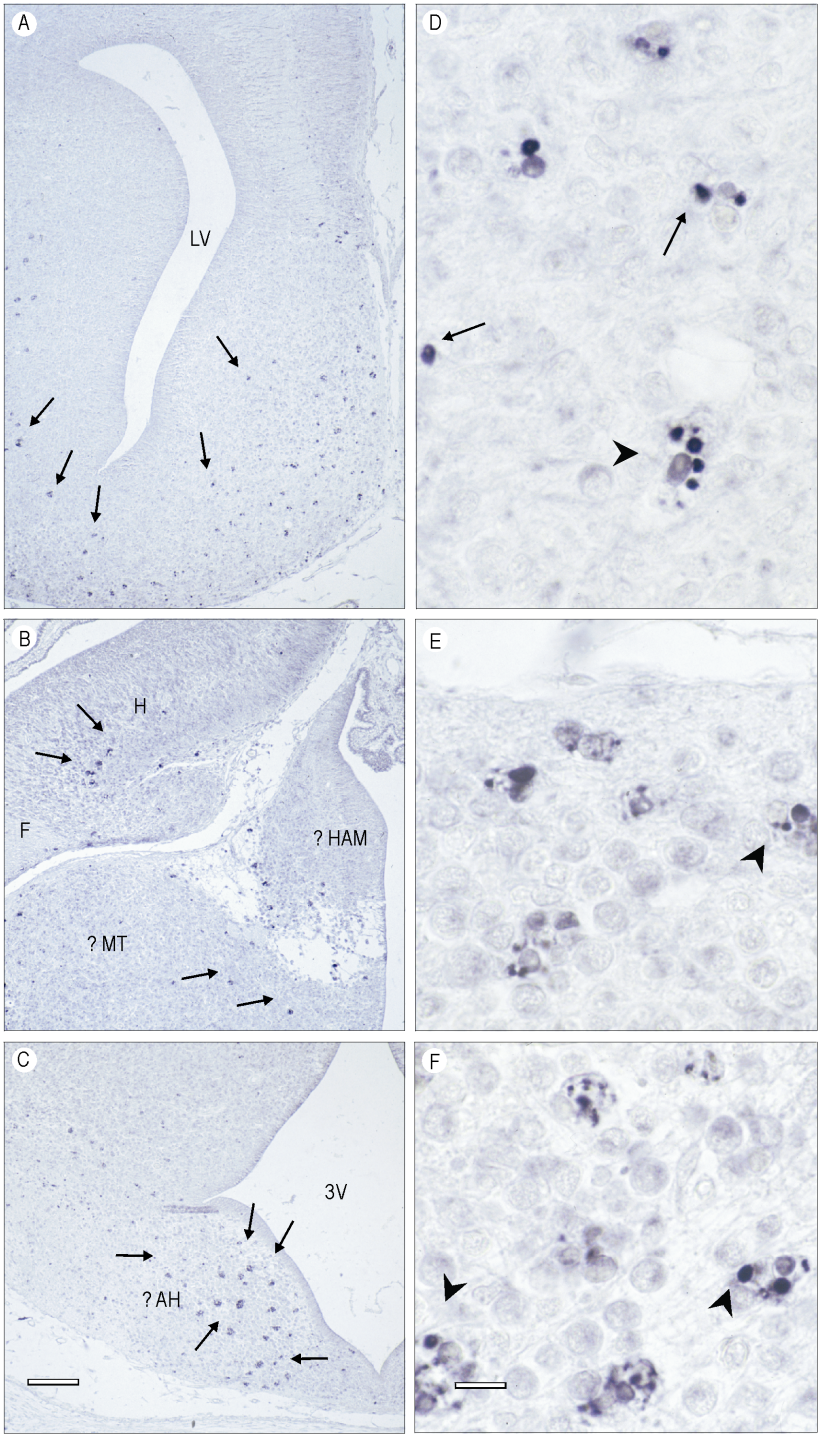


Fig.3) Low and high magnification micrographs of nick-end labelling of fragmented DNA (TUNEL) on coronal section of 18 days old munc 18-1 mutant (A-F) mouse embryos. Low magnification of the (**A**) anterior forebrain; (**B**) hippocampus (H) and putative medial thalamic nuclei (?MT); (**C**) third ventricle (3V) and putative anterior hypothalamus (?AH) with their respective high magnification on D, E and F. This staining (arrow) identified fragmented DNA in apoptotic bodies. Bar: 200μm. **D & F**) High magnification showing staining of either isolated (arrow) or grouped (arrowheads) apoptotic bodies. Grouped apoptotic bodies suggested that they were inside of a phagocyte. Bar: 20μm.

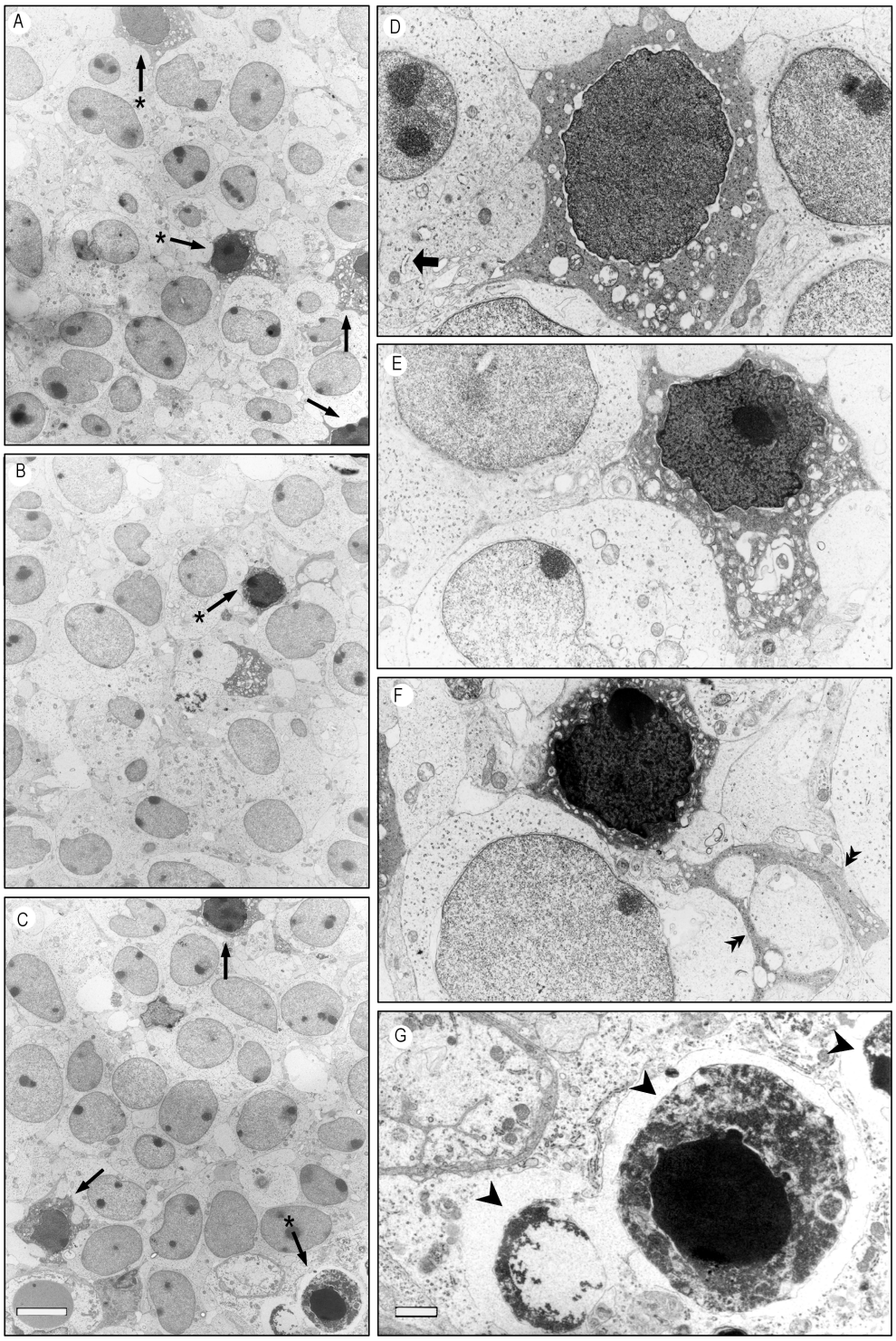


Fig.4) Electron mic-rographs of thalamic coronal sections of 18-day-old munc 18-1 mutant mouse em-bryos. **A-C**) Low ma-gnification overview. Neurones at diff-erent stages of ap-optosis (arrow) were electron denser (nu-cleus and cytopla-sm) than neighbou-ring neurones. Noti-ce the pleomorphic nuclei of the cell po-pulation. Asterisked arrows indicate cells that are magnified in the following photos. Bar: 5μm. **D**) Early apoptosis. The cell is the same size as its neighbourings, has increased prote-in synthesis (indica-ted by large lumen of the nuclear enve-lope), disrupted mi-tochondria, high nu-mber of intracellular vacuoles, visible ri-bosomes and in contact with neigh-bouring cells. The neighbouring neuro-nes were not fully differentiated (little rough endoplasmic reticulum (larger ar row) and no visible Golgi apparatus). **E & F**) At this stage, mitochondria in apoptotic cells could no longer be identified but nucleoli were still visible. These were shrunken but maintained contact with neighbouring cells. Notice the presence of cell processes (double arrowhead). **G**) Apoptotic cells fragmented onto apoptotic bodies (arrowhead), which contained degraded material. The nucleus was more heavily stained than in previous stages and no nucleoli were discernible. Bars: 1μm.

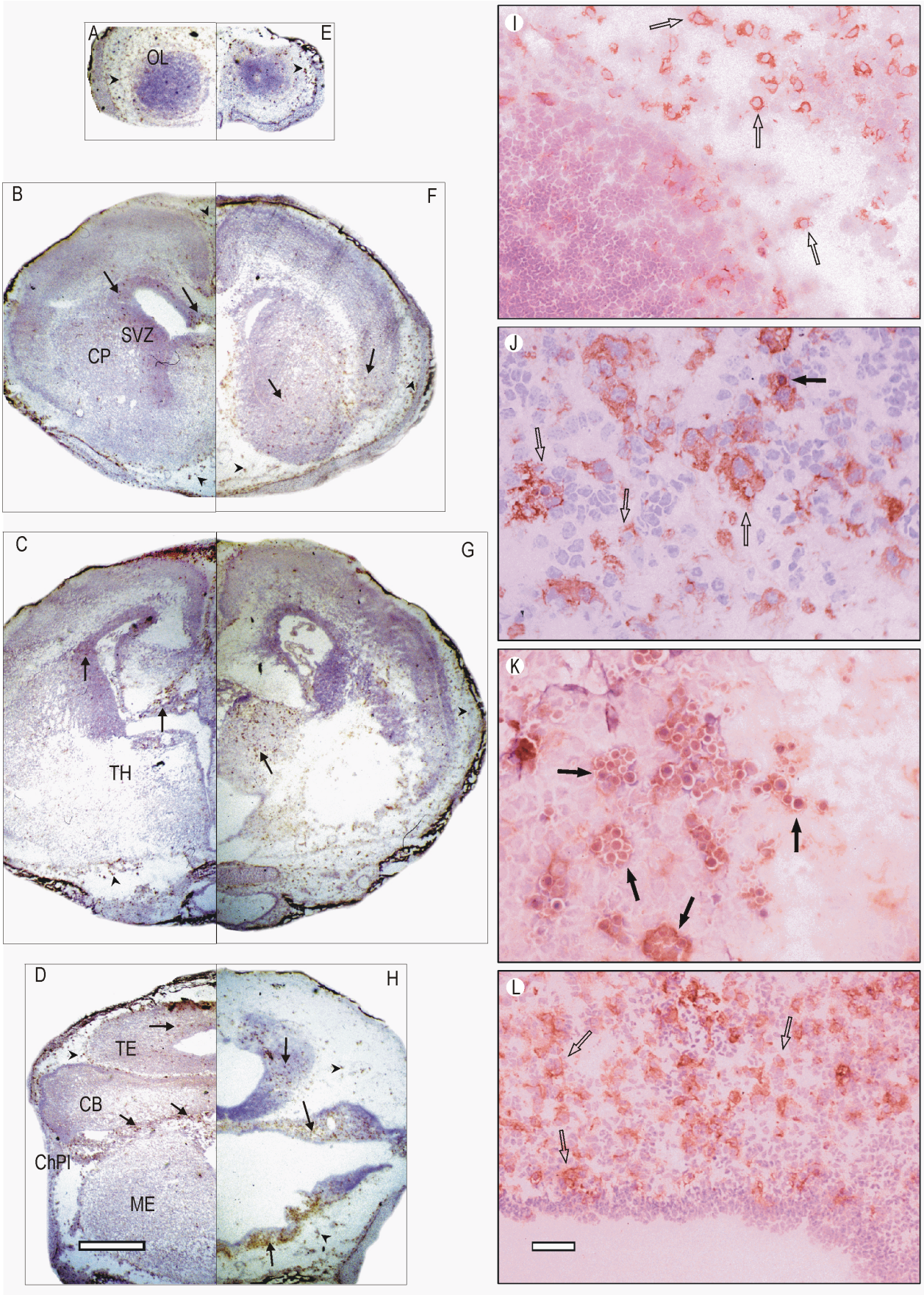


Fig.5) Micrographs of coronal sections from 18-day-old control (A-D) and munc 18-1 mutant (E-L) mouse embryos immunostained for the F4/80 macrophage protein. **A-H**) Low magnification micrographs of control (A-D) and mutant (E-H) em-

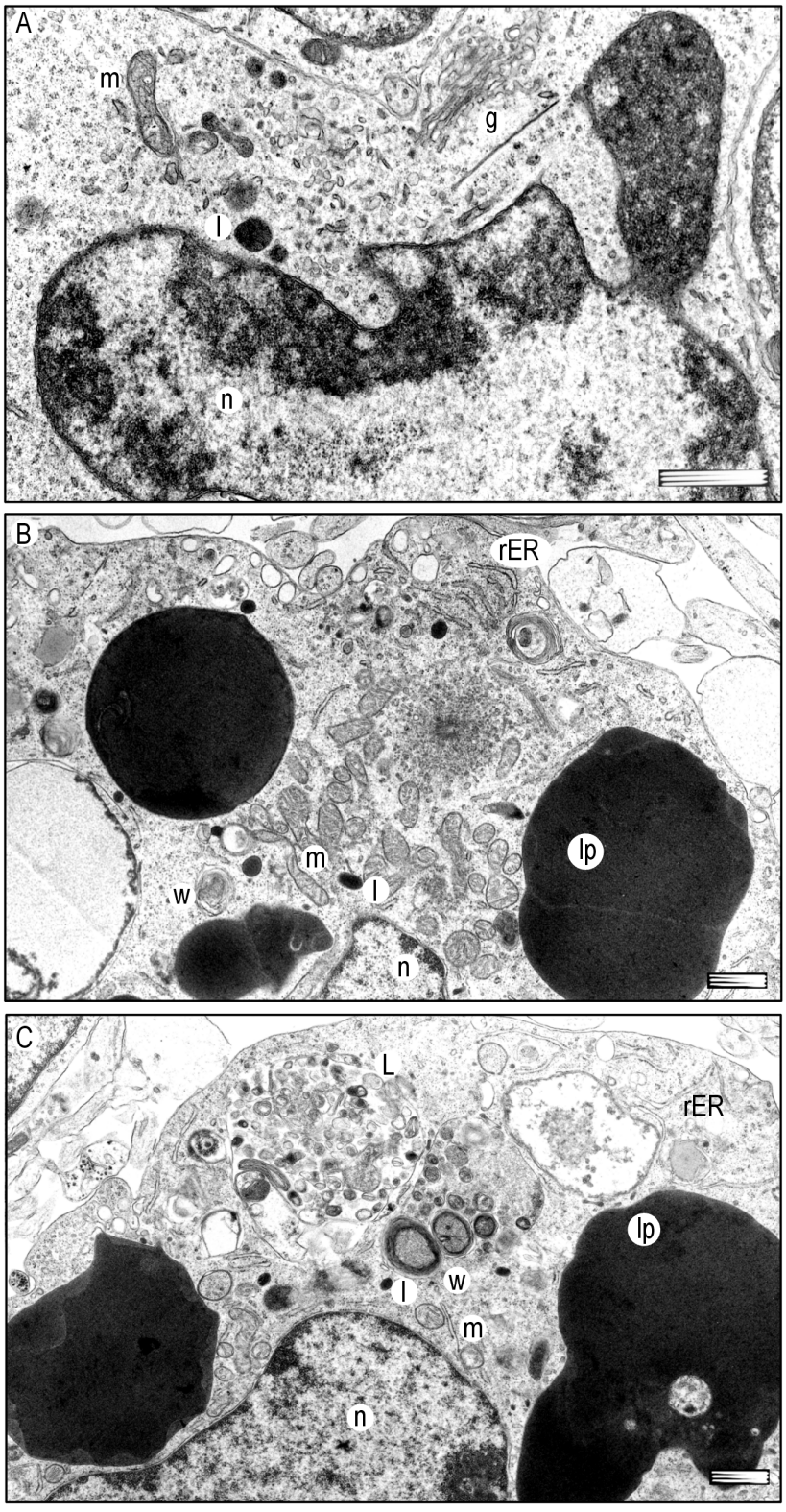


Fig.6) Electron micrographs of 18 day old munc 18-1 mutant embryo macrophages. **A**) Young inactive macrophage with nucleus (n), mitochondria (m), Golgi (g), primary lysosomes (l) and high number of ribosome rosettes and no rough endoplasmic reticulum (rER) or secondary lysosomes. **B & C**) Adult macrophages with primary (l) and secondary lysosomes (L), rER, mitocho-ndria (m), lipid droplets (ld) and nucleus (n). Those macrophages also had large amounts of multilamellar membranous whorls (w). Bars: 1μm.

bryos. F4/80$^+$ control cells were mostly localised outside of the brain tissue (arrowhead). The little staining inside of the brain tissue (arrow) occurred at the subventricular zone (SVZ), tectum (TE) and choroid plexus (ChPl). Mutants had abundant F4/80$^+$ macrophages both inside (arrow) and outside (arrowhead) the brain tissue. Mutant cerebellum and medulla were fully filled with F4/80$^+$ cells. Bar: 500μm. **I-L**) High magnification micrographs of mutant embryos at the (**I**) olfactory lobe (OL); (**J**) caudate putamen (CP); (**K**) thalamus (TH); (**L**) cerebellum (CB) showing macrophages containing (arrow) or not (empty arrows) phagocytosed debris. Bar: 30μm.

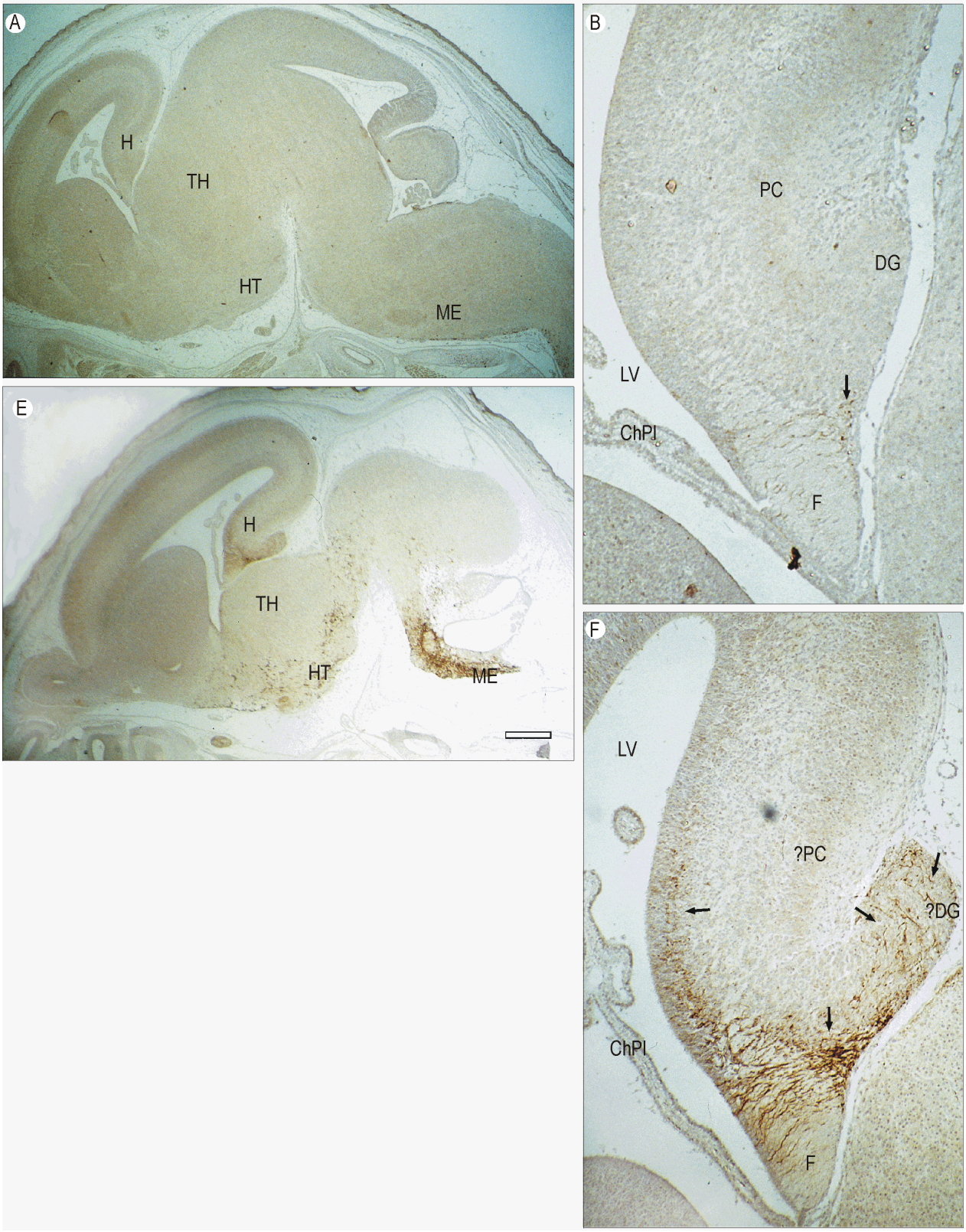
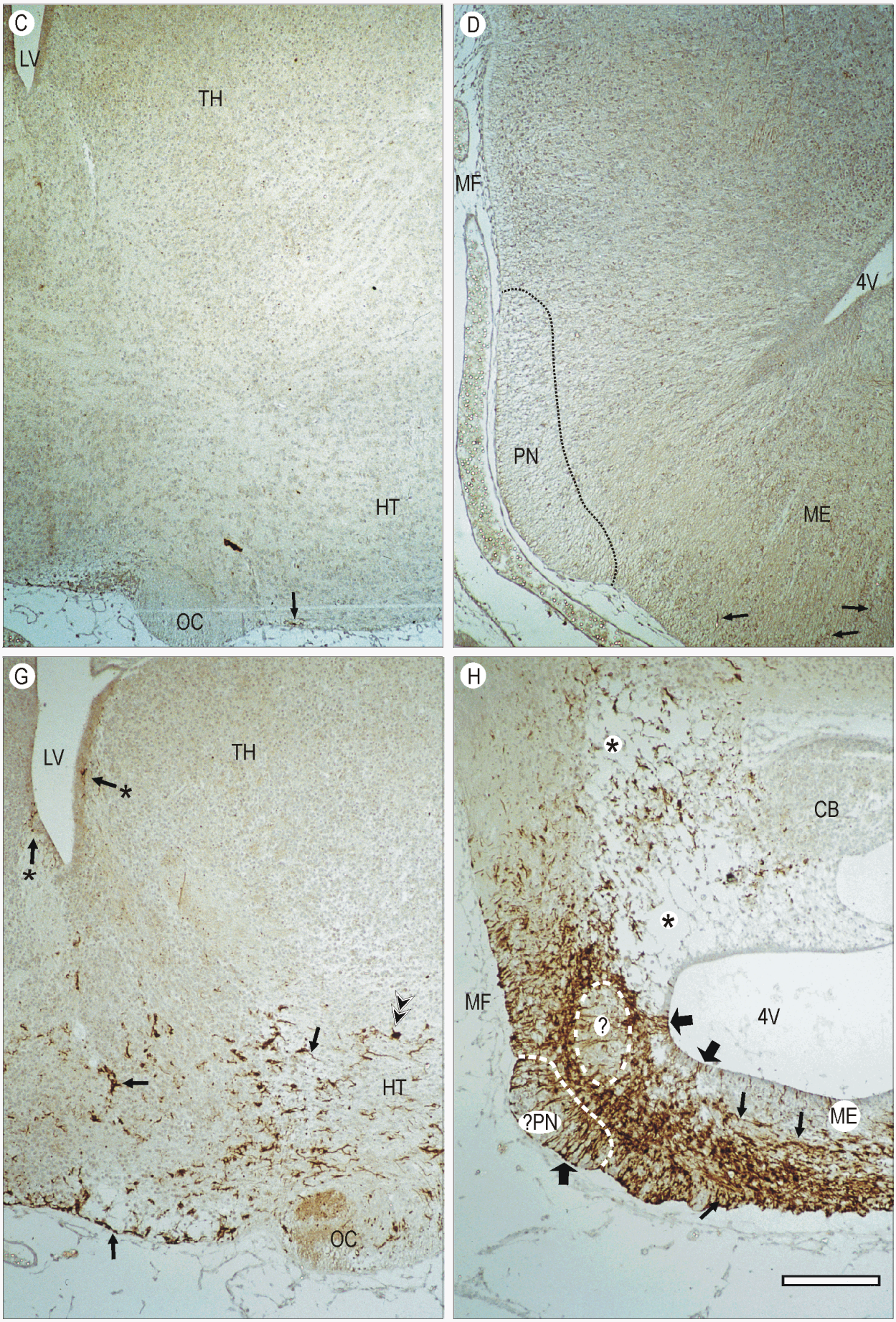


Fig.7) Micrographs of sagittal sections from 18 day old control (A-D) and munc 18-1 mutant (E-H) sibling mouse embryos immunostained for the astrocyte GFAP protein. **A & E**) Low magnification micrographs of control and mutant brains. GFAP$^+$ areas were observed in the hippocampus (H), the hypothalamus (HT), medulla (ME) and spinal cord. Bar: 500μm. **B & F**) High magnification of the hippocampal formation. Control embryos had only faintly stained dendrite astrocytes in the oriens layer (arrow). However, mutants had high numbers of GFAP$^+$ cells randomly scattered in the dentate gyrus (DG), in the oriens layer and in the fimbrae (F) among the fibres, but no positive cells in the presumptive pyramidal cell (?PC) layer. **C & G**) High magnification at the lateral ventricle (LV), thalamus (TH), hypothalamus (HT) and optic chiasma (OC). Control animals had no GFAP$^+$ cells in the thalamus or in the optic chiasma. A few dendrites occurred at the periphery of the ventral (followed page 79)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do mutants have a premature gliogenesis? A plausible explanation is that in mutants, the extensive apoptotic neuronal death removed neuronal repression on glial differentiation. Consequently, glial precursors initiated their differentiation. Alternatively, neuronal death induced massive phagocytosis that could, at least partially, be phagocytosed by astrocytes (Sturrock, 1981). However, because premature astrocytes differentiated in some foci, had a characteristic pattern, and appeared in areas where controls had little staining, it is unlikely that they were only induced for phagocytosis. This suggests that premature gliogenesis was not merely a phagocytosis reaction. Further, the activated macrophage staining pattern (Fig.5) was different from the astrocyte staining pattern (Fig.7). In the light of this, it is plausible that during development, neurones and glia co-ordinate their differentiation, at least partially via synapses.

It is known that CNS cells do not all differentiate simultaneously, e.g. the mitral cell layer of the olfactory lobe is formed before the cortex is layered. However, the factors co-ordinating this differentiation are less unclear. Evoked synaptic activity is not required for cell differentiation because the blockage of evoked secretion did not impair visual system or motor neurone differentiation (Harris, 1980; Seil and Drake-Baumann, 1995; Xie,H and Ziskind-Conhaim, 1995). We propose that spontaneous synaptic activity may be a check point factor for cell differentiation and survival.

(following page77 Fig.7) hypothalamus (arrow). Mutant animals had no staining in the thalamus except for few astrocytes bordering the lateral ventricle (asterisked arrow). However, the mutant hypothalamus had large numbers of positive cells (arrow), which were randomly scattered in the tissue and did not delimit any hypothalamic nuclei. Astrocyte dendrites were also seen in the optic chiasma among the fibres, as in the fimbrae. **D & H**) High magnification of the ponti nuclei (PN) and medulla (ME). Control animals had few $GFAP^+$ astrocytes in the ventral medulla (arrow). Mutant animals had extremely high amounts of $GFAP^+$ astrocytes, which were either parallel (arrow) or perpendicular (larger arrow) to the remaining fibres. Perpendicular cells sent dendrites to the fourth ventricle (4V) and mesencephalic flexus (MF). Here, those $GFAP^+$ cells were not randomly scattered but had a characteristic staining pattern, delimiting a non-identified brain nuclei (dotted line and ?) and positioned themselves along the fibres. Asterisk indicates missing cells. Bars: 200μm.

Concluding remarks

Summary of results

The aim of this thesis was to characterise the role of synaptic release in the development and maintenance of the mouse nervous system. For this, the munc18-1 mutant mouse was used as a model. Comparing wild type, heterozygous and homozygous animals first confirmed the role of munc18-1 protein in synaptic release. Heterozygous animals are viable, contain 50% of munc18-1 protein (compared to the wild type) and their synapses are able to transmit. Homozygous animals have no munc18-1 protein, are not viable but develop to term without any form of synaptic activity.

Heterozygous animals showed reduced spontaneous fusion of vesicles, reduced number of fused vesicles per stimulus, reduced response to hypertonic solutions and early fatigue at high frequency stimulation. From these observations, it was concluded that the munc18-1 protein is necessary for secretion by making synaptic vesicles accessible for fusion (chapter 2).

At birth, homozygous embryos were paralysed and died, probably due to breathing failure. Following embryonic development over time showed that the mutant CNS formed but subsequently degenerated. Neuronal wiring was normal and synapses were formed. Thus, the lack of synaptic activity was due neither to the lack of synaptic contact nor to the absence of synapses. Therefore, synaptic activity was apparently not necessary for the establishment of brain circuits but it was necessary for its maintenance (chapter 3).

At E16, the characterisation of synapses in the neocortex revealed that wild type and homozygous mutant animals had similar synapse morphology. Between E16 and E18, wild type synapses matured while mutant synapses did not. On both days, wild type animals had significantly more synapses than mutants. However at E18, mutants had more “multivesicular structures” than wild type animals. This suggests that mutant synapses at E16 were modified into “multivesicular structures” at E18, and synapses observed at E18 were novel synapses. Our results also suggest that a synapse with few vesicles matures only if it is functional (chapter 4).

At E18, anatomical analysis and glial staining showed that in the absence of synaptic activity, neurones died by apoptosis before they could become morphologically differentiated and glia differentiated earlier. TUNEL, macrophage staining and electron microscopy confirmed that cells died by apoptosis. Gliogenesis occurred prematurely with a similar pattern to the adult and in areas were neurones had degenerated. These results suggest that in the absence of functional synapses, neurones do not proceed with their differentiation but die by apoptosis and that gliogenesis occurs prematurely (chapter 5).

Munc18 protein and neuronal release

Previous reports suggested that munc18-1 and its *Drosophila* isoform, rop, function as negative regulators of vesicle fusion by binding to syntaxin protein (Pevsner et al., 1994a; Schulze et al., 1994; Wu et al., 1998). If munc18-1 is a negative regulator, synaptic release would not be impaired but increased after reducing munc18-1 protein. This was certainly not observed in the mice mutants. However, it is plausible that synaptic release in mutant animals was impaired because syntaxin and other synaptic proteins were expressed at low levels (chapter 2). Syntaxin is the plasma membrane partner for the core complex formation and is thought to be essential for synaptic vesicle fusion (Jahn and Sudhof, 1993; Sudhof, 1995; Bock and Scheller, 1999). But why were syntaxin and the other proteins expressed at low levels? One explanation is that munc18-1 protein functions as a chaperone protein “protecting” those proteins from

cellular proteases within protein complexes and facilitates their correct subcellular localisation, for instance in the axons and/or at the synaptic terminals. Another possibility is that the amount of those proteins is only increased upon functional synaptic contact. It was reported that the mRNA levels of synaptic proteins were regulated upon establishment of synaptic contacts (Lou and Bixby, 1995; Campagna et al., 1997).

Munc18-1 protein and cellular integrity

Knocking out the munc18-1 gene produced a lethal phenotype in mice and specific CNS cell groups underwent apoptosis. Mouse mutant neurones may have degenerated due to intracellular defects caused by the absence of munc18-1 protein. It was shown in transfected cells that overexpression of syntaxin without munc18-1 caused deformation of rough reticulum endoplasmic and Golgi (Rowe et al., 1999). However, such organelle deformation was not conspicuous in the mutant animals. Also contrasting with the cellular transfection experiments is the observation that in *Drosophila*, rop does not appear to localise to the endoplasmic reticulum or Golgi (Harrison et al., 1994). However, we cannot exclude the possibility that a biochemical defect, which does not result in a visible morphological deformation, caused the mutant neurone death. Alternatively, neurones may also have degenerated due to the lack of synaptic input. The evidence is that in mutants, almost all target neurones (these are neurones that receive afferent synaptic input) were degenerated and sensory neurones (these are neurones that innervate a target cell and do not receive afferent synaptic input), like those in the dorsal root ganglia, were not degenerated. Dorsal root ganglion neurones normally express munc18-1 protein (Bouwman and Verhage, unpublished data) and survive in mutants without munc18-1 protein.

The role of synaptic release in synaptogenesis

The role of neurotransmitters in embryonic development is a matter of debate (Lauder, 1988; Buznikov et al., 1996). Neurotransmitters have been implicated as morphogenetic factors during embryogenesis. However, our results show that if this is the case, neurotransmitters must be released via a munc18-1-independent mechanism. Our results also show that neurotransmitters are certainly necessary for nervous system maturation via munc18-1-dependent synaptic release but are not necessary for initial brain assembly.

It has been shown that synaptic release during postnatal development is essential for cell target survival and correct innervation. Unilateral nasal closure in the ferret significantly reduced the size of the ipsilateral olfactory lobe and the number of mitral cells (Cummings et al., 1997). Monocular deprivation during the sensitive period early in life, disrupts normal innervation of the visual cortex causing a decrease in the number of cortical cells responding to stimulation from the deprived eye (Chapman et al., 1986) (for review see Wiesel, 1982). From this, it was concluded that sensory activity in these systems is necessary for neuronal survival and correct target innervation. Synaptic release has also been implicated in refining synaptic contacts by co-ordinating neuronal competition. In the NMJ, postsynaptic activation via acetylcholine release initiates two types of signals: one protective and another destabilising. The protective signal is intrasynaptic and specific to the innervating axon and postsynaptic receptors. The destabilising signal is an intersynaptic “punishment” that propagates within the postsynaptic cell and allows active synapses to destabilise others that are inactive. This punishment signal decreases in potency with distance (for review see Jennings, 1994; Lichtman and Colman, 2000). Competition for the innervation of the lateral geniculate nucleus neurones by retinal neurones from both eyes may also happen in an synaptic activity-

dependent manner. This activity happens spontaneously before the onset of visual experience (Penn et al., 1998). Taken together, these experiments reveal that synaptic activity is not necessary for synapse formation but is essential for synaptic contact refinement, synaptic competition and to a certain extent, for target development.

Such types of experiments examining evoked secretion did not block spontaneous secretion and furthermore dealt with synapses that were already formed. In munc18-1 mutants, action potential is not impaired but still their synapses were unable to secrete. In those mutants evoked and spontaneous synaptic secretion were blocked and were unnecessary for initial synapse formation and for brain assembly. From the munc18-1 mutant model, it is suggested for the first time that before synaptic maturation and competition, synaptic activity is necessary for synapse maintenance and that synapses can be eliminated without synaptic secretion (chapter 4). However the process of synapse elimination in absence of synaptic activity is unlikely to be the same as the activity-dependent synapse elimination described to occur at the NMJ (Lichtman and Colman, 2000).

The role of spontaneous synaptic release

By means of *in vitro* experiments, spontaneous synaptic release was hypothesised to have a role in synapse maturation (Beals, 1976; Seil and Drake-Baumann, 1995). However, the role of spontaneous synaptic release in the nervous system has been scarcely demonstrated. This is particularly due to the lack of a model system, as well as the lack of pharmacological drugs disabling synaptic vesicle fusion. Spontaneous neurotransmitter release was abolished in munc18-1 homozygous mutant animals.

Growing axons spontaneously secrete neurotransmitters, such as acetylcholine and GABA, via an unknown vesicular mechanism before synaptic formation (Xie and Poo, 1986; Sun and Poo, 1987; Gao and Van Den Pol, 2000). This process may have a role in synaptogenesis, neurite growth, cell division, migration and survival (LoTurco et al., 1995; Barker et al., 1998). Several *in vitro* experiments revealed that synapse silencing by blocking action potential and receptors did not impair normal neuritic network formation (Van Huizen et al., 1985; Verderio et al., 1994). Other experiments reported that synapse silencing by syntaxin cleavage caused cell death (Williamson and Neale, 1998) and blocking the glutamate receptor *in vivo* caused massive apoptotic cell death (Ikonomidou et al., 1999). It is plausible that the basic protein machinery for membrane fusion is unaltered in munc18-1 mutants and the munc18-1 protein acts specifically in synaptic fusion. This is supported by the findings that secretory paths have specific protein adapters, for instance the caps protein involved in LDCV but not in synaptic vesicle fusion (Berwin et al., 1998).

In this thesis, some consequences of abolishing synaptic activity for nervous system development are reported. The results suggest that munc18-1-dependent fusion is not necessary for brain assembly, axonal outgrowth, cell migration or synapse formation. The results also suggest that during normal development without synaptic release a “basic synapse” is formed. Upon synaptic release, synapses mature and co-ordinate cell differentiation. It is only then that experience-induced synaptic refinement (synapse elimination and reformation) will remodel and mature the synaptic networks, via synaptic competition.

Future research

Mutant synapses are initially morphologically normal but are not functional. This facilitates the study of the

role of synaptic activity during embryonic development. However, the major point that still needs to be confirmed is if neuronal death in mutants is caused by the lack of synaptic release. Co-culture of wild type together with munc18-1 mutant neurones will bring together the establishment of synaptic contacts between functional and non-functional neurones. If munc18-1 neurones cannot survive under these conditions, an intracellular biochemical defect is likely the cause of mutant cell death. If munc18-1 neurones can survive in these experimental conditions, it would mean that the cause of neuronal cell death is indeed the lack of synaptic release. Recent results have shown that this co-culture has prolonged the numbers of days of mutant neurones *in vitro* (Heeroma, Van Aerde and Verhage, in preparation). Synapse formation and elimination will also be followed in this *in vitro* system.

Is it possible that the induction of genes follow a spatial and temporal pattern expression as a result of synapse activity? There is some evidence that this is the case. It was reported in chick ciliary ganglion that synaptophysin mRNA levels increased at the time of neurone-target contact. In contrast, acetylcholine transferase mRNA levels increased at the time of synaptic maturation (Lou and Bixby, 1995; see also Campagna et al., 1997; Plunkett et al., 1998). The munc18-1 mutant mice offer the possibility of identifying specific genes that might be expressed before functional synaptic contact and during synapse maturation. Comparing mRNA from mutant (whose synapses do not mature) with those from wild type (whose synapses do mature) will allow a regional and temporal collection of genes involved in synapse maturation and consequently in neuronal survival.

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Nederlandse samenvatting

Het doel van dit proefschrift was het karakteriseren van de rol die neurotransmitter speelt in de ontwikkeling en het stabiliseren van het zenuwstelsel in de muis. Hiertoe is de Munc18-1 deficiente muis als modelsysteem gebruikt. Allereerst bevestigde een vergelijking van wildtype, heterozygote en homozygote munc18-1 deficiente muizen de rol van munc18-1 in synaptische secretie. Heterozygote dieren zijn levensvatbaar, bevatten 50% munc18-1 eiwit (vergeleken met wildtype dieren) en hebben synapsen die in staat zijn tot neurotransmitter secretie. Homozygote dieren hebben geen munc18-1 eiwit en zijn niet levensvatbaar, maar ontwikkelen zich wel tot aan de geboorte zonder enige vorm van synaptische activiteit.

Heterozygote dieren lieten minder spontane fusie en minder gestimuleerde fusie van neurotransmitter blaasjes zien alsmede een verlaagde respons op hypertone oplossingen en versnelde afzwakking van secretie bij hoogfrequente stimulatie. Uit deze bevindingen is geconcludeerd dat het munc18-1 eiwit nodig is voor secretie daar het synaptische blaasjes toegangkelijk maakt voor fusie (hoofdstuk 2).

Homozygote dieren waren verlamd en stierven bij geboorte, vermoedelijk door gebrek aan ademhaling. Analyse van embryonale ontwikkeling door de tijd liet zien dat het mutante centrale zenuwstelsel werd gevormd maar vervolgens weer degenereerde. Neuronale connectiviteit was normaal en synapsen werden gevormd en daarom was het gebrek aan synaptische activiteit niet het gevolg van een gebrek aan neuronal uitgroei of het gebrek aan synapsen. Kennelijk was synaptische activiteit niet zozeer noodzakelijk voor het aanleggen van neuronale contacten in het brein, maar eerder voor het in stand houden hiervan (hoofdstuk 3).

Op 16 ontwikkeling dag 16 (E16) gaf analyse van synapsen van de neocortex aan dat wildtype en homozygoot mutante dieren een vergelijkbare synapsmorfologie hadden. Tussen E16 en E18 ontwikkelden wildtype synapsen zich verder terwijl mutante synapsen dat niet deden. Op beide tijdspunten hadden wildtype dieren significant meer synapsen dat mutanten. Mutante dieren hadden daarentegen meer “multivesiculaire structuren” op E18. Dit suggereert dat de mutante synapsen van E16 werden aangepast tot “multivesiculaire structuren” op E18, en dat de synapsen die op E18 gezien werden nieuw waren. Onze resultaten geven ook aan dat een synaps die een paar synaptische blaasjes heeft zich alleen tot volwassenheid kan ontwikkelen wanneer het functioneel is (hoofdstuk 4).

Anatomische analyse en gliële kleuring op E18 lieten zien dat neuronen stierven door apoptose voordat ze zich morfologisch konden differentiëren wanneer er geen synaptische activiteit is. Bovendien differentiëren glia zich eerder. TUNEL, macrofaag kleuring en electronen microscopie bevestigden dat cellen stierven door apoptose. Gliogenese vond voortijdig plaats op plekken waar neuronen gedegenereerd waren en met een patroon lijkend op dat in volwassenen. Deze resultaten geven aan dat, in de afwezigheid van fucntionele synapsen, neuronen niet verder differentiëren maar sterven door apoptose (hoofdstuk 5).

Résumé en français

L'objectif de cette thèse a été de caractériser le rôle de la transmission synaptique dans le développement du système nerveux central (CNS) de la souris. Nous avons utilisé comme modèle la souris mutante pour la protéine munc18-1. Cette protéine joue un rôle essentiel dans la transmission synaptique comme le montre la comparaison entre animaux normaux, hétérozygotes et homozygotes pour la mutation Chez les souris hétérozygotes, contenant seulement 50% de la protéine munc18-1, les synapses sont fonctionnelles. Et ces animaux sont viables. Par contre, chez les souris homozygotes qui ne synthétisent aucune protéine munc18-1, il n'existe aucune forme de transmission synaptique. Ces animaux ne sont pas viables. Par contre, ils se développent jusqu'à terme. Chez les souris hétérozygotes, les synapses présentent une réduction à la fois de la fusion spontanée des vésicules synaptiques avec la membrane pre-synaptique, du nombre de vésicules fusionnant par stimulus et de la réponse à des solutions hypertoniques. Il existe aussi une "fatigue" précoce quand ces synapses sont stimulées à hautes fréquences (chapitre 2).

Les embryons homozygotes sont paralysés et meurent à la naissance, probablement à la suite d'une insuffisance respiratoire. Au début de la vie embryonnaire, le SNC de ces souris mutantes se développe normalement. Les axones et les dendrites sont normaux et des synapses se forment. Le manque de transmission synaptique observée ne peut donc pas être dû à une absence de connexion cérébrale. Cependant, ce SNC dégénère par la suite. Apparemment, l'activité synaptique n'est pas nécessaire à la formation des connexions cérébrales mais plutôt à leur maintien (chapitre 3).

L'étude des synapses du cortex au 16ème jour de la vie embryonnaire (E16) montre que les animaux normaux et homozygotes possèdent des synapses identiques. Cependant, entre E16 et E18, les synapses chez les souris normales mûrissent contrairement à celles des animaux mutants. Aussi bien à E16 qu'à E18, les animaux normaux possèdent plus de synapses que les mutants. Par contre, à E18, les souris mutantes ont plus de "structures multivésiculaires" que les animaux normaux. Une interprétation possible de ces résultats est que les synapses retrouvées chez les mutants à E16 évoluent en "structures multivésiculaires" et que les synapses observées à E18 sont nouvellement formées. Nos résultats suggèrent aussi que les premières synapses formées avec quelques vésicules ne peuvent mûrir que si elles sont fonctionnelles (chapitre 4).

À E18, en l'absence d'activité synaptique les neurones meurent par mort cellulaire programmée (apoptose) avant d'être morphologiquement différenciés alors que les cellules gliales se différencient prématurément. TUNEL, immunomarquage des macrophages et microscopie électronique confirment que les cellules meurent par apoptose. Différenciée prématurément, les cellules gliales présentent un motif de marquage ressemblant à la situation adulte dans les aires où les neurones ont disparu. Ces résultats suggèrent qu'en l'absence de transmission synaptique, les neurones ne peuvent continuer à se différencier et meurent par apoptose (chapitre 5).

Resumen en español

El objetivo de esta tesis es la caracterización del papel de la liberación sináptica en el desarrollo y mantenimiento del sistema nervioso de ratón. Para ello, el ratón mutante para la proteína munc 18-1 ha sido usado como modelo. Tras la comparación con el ratón salvaje, los animales heterocigotos y homocigotos confirmaron el papel de la proteína munc 18-1 en la liberación sináptica. Los animales heterocigotos son viables, contienen un 50% de proteína munc 18-1 (respecto al salvaje), y sus sinapsis son capaces de transmitir. Los animales homocigotos no tienen proteína munc18-1 y no son viables, pero son capaces de desarrollarse plenamente, aunque carecen de cualquier forma de transmisión sináptica.

Los animales heterocigotos mostraron una disminución en la fusión espontánea de vesículas, un numero reducido de vesículas de fusión por estímulo, una disminución en la respuesta a soluciones hipertónicas, y síntomas de fatiga temprana tras estimulación de alta frecuencia. A partir de estas observaciones, se concluyó que la proteína munc 18-1 es necesaria para la secreción en la medida en que posibilita que las vesículas sinápticas sean accesibles para la fusión (capítulo 2).

En el momento del nacimiento, los embriones homocigotos sufren parálisis y mueren, probablemente debido a fallos respiratorios. El seguimiento del desarrollo embrionario a lo largo del tiempo demostró que el sistema nervioso central de los ratones mutantes se desarrolla con normalidad, pero que posteriormente degenera. La arborización sináptica y el establecimiento de sinapsis son normales. Por tanto, se confirma que la falta de actividad sináptica no es debida a la ausencia de contacto sináptico o de sinapsis. Esto significa que, aparentemente, la actividad sináptica no es necesaria para el establecimiento de circuitos cerebrales, pero sí para su mantenimiento (capítulo 3).

La caracterización de sinapsis en el neocortex a día embrionario E16 (E16), reveló que los animales salvajes y mutantes presentan una morfología sináptica parecida. Entre E16 y E 18 las sinapsis salvajes maduran, pero no las mutantes. No obstante, a E18 los animales mutantes presentaban mas “estructuras multivesiculares” que los salvajes. Esto sugiere que las sinapsis mutantes a E16 se transformaron en “estructuras multivesiculares” a E 18, y que las sinapsis observadas a E18 son nuevas sinapsis. Nuestros resultados también sugieren que una sinapsis con pocas vesículas madura únicamente si es funcional (capítulo 4).

El análisis anatómico y la tinción glial a E18 revelaron que, en ausencia de actividad sináptica las neuronas mueren por apoptosis antes de poder alcanzar un estado de diferenciación morfológica, y que se produce una diferenciación glial prematura. Mediante técnicas de TUNEL, tinción de macrófagos y microscopía electrónica se confirmó que las células mueren por apoptosis. La gliogénesis ocurre de forma prematura, presentando un patrón parecido al del adulto y en áreas en las que las neuronas habían degenerado. Estos resultados sugieren que, en ausencia de sinapsis funcionales, las neuronas no continúan diferenciándose y acaban muriendo por apoptosis (capítulo 5).

Resumo em português

O objetivo desta tese foi caracterizar o papel da transmissão sináptica no desenvolvimento e na manutenção do sistema nervoso de camundongo. Para isso, o camundongo mutante munc18-1 foi usado como modelo. A Comparação entre os animais de tipo selvagem, heterozigotos e homozigotos confirmou o papel da proteína munc18-1 na transmissão sináptica. Os animais heterozigotos, os quais contêm 50% de proteína munc18-1 (comparado com o tipo selvagem) são viáveis, e suas sinapses são funcionais. Os animais homozigotos não sintetizam a proteína munc18-1, não são viáveis e se desenvolvem até a nascência sem nenhuma forma de atividade sináptica.

Os animais heterozigotos apresentaram redução da fusão espontânea das vesículas sinápticas reduzida e das vesículas fusionadas por estímulo, uma resposta reduzida à soluções hipertônicas e uma fadiga precoce a estímulo a alta freqüência . Dessas observações, foi concluído que a proteína munc18-1 é necessária à transmissão sináptica, pois torna as vesículas acessíveis à fusão (capítulo 2).

Ao nascer, os embriões homozigotos estavam paralisados e morreram, provavelmente devido a uma insuficiência respiratória. Seguindo-se o desenvolvimento embrionário a diferente tempos de gestação, pôde-se ver que o sistema nervoso central dos mutantes foi formado, porém em seguida degenerou-se. Os axônios e os dendritos eram normais e as sinapses foram formadas o que indica que a ausência de atividade sináptica observada não foi devida à falta de contato sináptico. Aparentemente, a atividade sináptica não é necessária ao estabelecimento de circuitos cerebrais, mas sim à sua manutenção (capítulo 3).

No 16° dia de gestação (E16), a caracterização de sinapses no córtex revelou que o tipo selvagem e os animais mutantes homozigotos possuíam sinapses semelhantes. Entre os E16 e E18, as sinapses de tipo selvagem amadureceram, contrariamente às sinapses mutantes. Em ambos dias, os animais de tipo selvagem tiveram significativamente mais sinapses que os mutantes. No E18, portanto, os mutantes tiveram mais “estruturas multivesiculares” que os animais de tipo selvagem. Isto sugere que as sinapses mutantes no E16 foram modificadas em “estruturas multivesiculares” no E18 e que as sinapses mutantes observadas no E18 eram sinapses formadas neste mesmo dia. Nossos resultados sugerem também que as primeiras sinapses formadas com poucas vesículas somente amadurecem se forem funcionais (capítulo 4).

No E18, análises anatômicas e colorações de células glia mostraram que em ausência de atividade sináptica, os neurônios morreram por apoptosis antes de tornar-se morfologicamente diferenciados e as células glia diferenciaram-se antes do tempo. A utilização da técnica do TUNEL, colorações macrófagas e microscopia eletrônica confirmaram que as células morreram por apoptosis. A formação das células glia ocorreu de forma prematura, com um padrão similar a do adulto e em áreas nas quais os neurônios tinham se degenerado. Estes resultados sugerem que em ausência de sinapses funcionais, os neurônios não continuam suas diferenciações mas morrem por apoptosis (capítulo 5).

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Curriculum Vitae

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